VOLUME I

Basic Aspects

Basic Aspects

DEDICATION

We dedicate this book to the Organizing Committee: E. Johannison, P. Melzer, H.S. Jacobs, P.C. Sizonenko, F.H. Schroder and F. Comite and to all our colleagues whose work with GnRH analogues is increasing the quality of life for individuals suffering the wide range of medical conditions benefited by the use of these agents.

B. H. Vickery
B. Lunenfeld

VOLUME I

GnRH ANALOGUES IN CANCER AND HUMAN REPRODUCTION

Basic Aspects

Edited by
B. H. Vickery and B. Lunenfeld

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Volume III. Benign and Malignant Tumors

Volume IV. Precocious Puberty, Contraception, Safety Issues

PREFACE

These four volumes comprising "GnRH Analogues in Cancer and Human Reproduction" are a distillation of the presentations of the invited speakers at a landmark International Symposium bearing the same name, organized by one of us (B.L.) and held in Geneva, Switzerland in February 1988. The Symposium was truly interdisciplinary spanning gonadal hormone dependent disease including various forms of cancer and ranging to control of fertility, both pro- and conception. The international flavor can be caught from the 480 participants and 259 contributors drawn from 14 countries. The Symposium, and therefore this book, would not have been possible without the backing of The International Committee for Research in Reproduction and the sponsorship of the International Society of Gynecologic Endocrinology, The Swiss Society of Fertility and Sterility, The University of Geneva School of Medicine, The Swiss Society of Endocrinology and The US Foundation for Studies in Reproduction Inc., and help from the World Health Organization.

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INTRODUCTION

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Since 1971 and the isolation, identification and synthesis of mammalian GnRH by Drs. Schally and Guillemin the pace of research in this subject area has been explosive and involved many disciplines. Synthetic efforts have produced several thousand analogues; seven of the most potent agonists are under commercial sponsorship and are either marketed or in late stages of clinical trial around the world. Prostatic cancer, precocious puberty and endometriosis patients now have a valuable new therapy to alleviate their condition and progress. A range of other applications is summarized in Chapter 1 and Volumes 2-4.

Basic Aspects

The chemistry of the nona- and decapeptide agonist analogues (Chapter 2) appears to have matured and no advances in potency have been reported since the early 1980s. An intriguing new direction for chemistry may follow the reports (Chapter 3) on new reduced size analogues which may be tailored to agonism or antagonism, at least in vitro. If high potency could be achieved in vivo these hexapeptides could potentially lead to the first orally administered agents, assuming stability to gastro-intestinal degradation. We are finally beginning to understand the molecular biology of GnRH (Chapter 4) and this in turn may suggest new chemical directions. The controversy over extrapituitary receptors and sites of action of GnRH in animals and man continues (Chapters 5 & 10); early reports of direct inhibitory effects of GnRH agonists on breast cancer cells seem to have given way to evaluation of the antagonists for this purpose. We have progressed through several generations of GnRH competitive antagonist analogues (Chapters 6-8) and in this iterative fashion may finally be approaching molecules which retain sufficient potency, while minimizing adverse effects, to be clinically evaluable. As noted, presently available analogues of GnRH are not deliverable orally but great progress has been made with alternatives to daily injection, first by intranasal insufflation

and then the potentially more compliance-compatible 1-3 month depot injectable formulations (Chapters 9, 11-12).

Reproduction and Gynecology

The utility of GnRH agonists in the management of endometriosis and their superior acceptability to existing approved therapy appears amply demonstrated, at least in so far as pain and size/extent of lesions are concerned (Chapters 13-18). Whether this, or indeed any other form of management, increases fertility outcome is still open to debate. To the extent that the GnRH agonists shut down ovarian function they are useful in symptomatic relief of dysfunctional bleeding and polycystic ovarian disease (Chapters 19 and 25). Correction of these conditions beyond the period of treatment however is not likely. A triphasic regimen, consisting of a GnRH agonist prior to and/or in conjunction with exogenous gonadotropins is being used in primary, idiopathic infertility, particularly in clomiphene failure and in secondary infertility such as polycystic ovarian disease, oligomenorrhea and premature menopause induced by chemotherapy. In these cases the suppression of endogenous gonadotropins with the GnRH analogue makes it easier to control follicular development and also eliminates the problems of premature and asynchronous luteinization (Chapters 22-24). This regimen also is being evaluated for oocyte harvest in IVF/ET programs (Chapters 28, 29).

Management of Tumors of the Reproductive System

A wealth of evidence now indicates the GnRH agonists to be equivalent to orchiectomy in palliation of metastatic prostatic cancer (Chapters 40, 43). Problems associated with the flare or initial stimulatory effect of the analogues and possibly to continued adrenal androgen secretion, a concept initially championed by Labrie, are receiving wide geographical attention (Chapters 39-42, 44). It seems that "total androgen blockade" by combination of a GnRH agonist and an antiandrogen may slightly delay time to progression of disease but do little for survival figures, with the notable exception of the results from one group. Breast cancer, ovarian cancer, uterine leiomyomata and BPH are all receiving attention although the numbers of cases so far reported are few (Chapters 31-37, 45-48). Interestingly, on the basis of animal studies, some tumor types such as pancreatic acinar and osteosarcomas may need to be redefined as to their hormonal dependency. Animal studies also suggest that coadministration of GnRH analogues with representatives of another new peptide series, the somatostatin analogues, may be beneficial for treatment of certain tumors (Chapter 49).

Precocious Puberty

The relatively rare but relatively devastating syndrome of central or true isosexual precocious puberty may now be treatable on more than a synmptomatic level. Several studies with the GnRH agonists are now of sufficient duration to suggest that a major, positive influence on final adult height may result from long term treatment (Chapters 53-57).

Safety

As always, with any new class of therapeutic agents, safety considerations must be paramount. Potent endocrine drugs such as the GnRH analogues should not be expected to be without "side effects". These unwanted effects will include sequellae of the mechanism of action and reflect the induction of a hypogonadotropic hypogonadism, such as vasomotor symptoms, in both sexes and osteoporotic changes in women, together with changes in gonadotropin subunit secretion (Chapters 51, 58). Clearly in this situation, benefit - to - risk considerations, as far as they can be assessed, will play a role (Chapters 67, 68). In so far as established therapy already includes extirpation of the gonads, no additional risk would appear to ensure from the use of GnRH agonists. The situation with the antagonistic analogues of GnRH thus far is more complex, involving the additional factor of an anaphylactoid response. Additional chemical efforts are required before these agents are to be considered for widespread trial, let alone therapeutic candidates.

The geographic and linguistic diversity of the contributors to this book together with constraints of early publication resulted in a somewhat autocratic editorial policy. The contributors are to be congratulated on their hard work; any inadvertent omissions or changed emphasis or context are the responsibility of the editors.

1

ANALOGS OF LHRH: THE PRESENT AND THE FUTURE

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INTRODUCTION

Seventeen years have passed since our laboratory accomplished the isolation, determination of structure and synthesis of the hypothalamic hormone controlling the secretion of both LH and FSH from the anterior pituitary gland [1-6]. This hypothalamic hormone is known as the LH and FSH releasing hormone, (LHRH/FSHRH) or gonadotropin-releasing hormone (GnRH) [6, 7]. While LHRH is also established as the main FSH releasing hormone, for reasons of convenience and historical continuity, the abbreviation LHRH is generally used for naming its analogs.

Many studies demonstrated that LHRH is the main link between the brain and the pituitary gland insofar as reproductive function is concerned [5-7]. Various clinicians, particularly our collaborators, established clinical uses of LHRH [8-13]. In 1971, we also postulated that LHRH or its analogs might be useful for the control of fertility by disrupting the menstrual cycle and preventing the ovulatory LH surge [14]. We suggested that replacement of one or more amino acids in LHRH might result in analogs possessing features required for binding to receptors, but devoid of intrinsic activity. Since 1972, systematic work has been proceeding to synthesize agonistic and antagonistic analogs of LHRH. A strong interest in medical applications of LHRH derivatives stimulated this undertaking. In the past 16 years, more than 2000 analogs of LHRH have been synthesized. Agonistic analogs more potent than the parent hormone have been made. Many of these analogs are being used clinically (Table 1) and the list of their applications is steadily expanding.

Various investigators have determined that large doses of superactive stimulatory analogs of LHRH cause paradoxical antifertility effects in animals and human being. Extensive work is in progress to apply these effects to the development of new contraceptive methods. The phenomena of pituitary desensitization and inhibition of sex steroid levels by LHRH agonists are being used for treatment of true idiopathic precocious puberty, endometriosis, leiomyomas and benign prostatic hyperplasia (BPH). Chronic administration of LHRH agonists is being utilized to

induce the regression of endocrine-dependent malignant neoplasms, especially prostate and breast cancer and more recently, ovarian carcinoma. LHRH agonists are also being tried in IVF-ET, GIFT and polycystic ovarian disease.

TABLE I

LH-RH AGONISTS IN CLINICAL USE

	STRUCTURE										RELATIVE POTENCY	ROUTE OF ADMINISTRATION
	1	2	3	4	5	6	7	8	9	10	1	—
LH-RH	p- Glu	- His	- Trp	- Ser	- Tyr	- Gly	- Leu	- Arg	- Pro	- Gly-NH_2		
Buserelin (Hoechst)	———	———	———	———	———	D-Ser(TBU)	———	———	———	Ethylamide	100	SC, IN, DEPOT
Nafarelin (Syntex)	———	———	———	———	———	D-(2-Nal)	———	———	———	———	200	SC, IN, DEPOT
Leuprolide (Abbott-Takeda)	———	———	———	———	———	D-Leu	———	———	———	Ethylamide	50	SC, IN
Lutrelin (Wyeth)	———	———	———	———	———	D-Trp-7-N-Me-Leu	———	———	———	Ethylamide	100	
Goserelin (ICI)	———	———	———	———	———	D-Ser(TBU)	———	———	———	Az-Gly-NH_2	50	SC, DEPOT
Histrelin (Ortho)	———	———	———	———	———	D-His(Bzl)	———	———	———	Ethylamide	100(?)	
Decapeptyl (Debiopharm)	———	———	———	———	———	D-Trp	———	———	———	———	100	SC OR DEPOT

SC, subcutaneous; IN, intranasal; DEPOT-IM, intramuscular for once a month microcapsules of for implants

Potent inhibitory analogs of LHRH, which block ovulation in laboratory animals, have been synthesized. Several antagonists of LHRH have been tested in men and women and shown to be active enough for practical use. The approach based on inhibitory analogs of LHRH has clearly been proved feasible for the development of new methods of birth control, although the exact clinical regimens are still lacking. In addition, it is possible that the LHRH antagonists could be useful for treatment of precocious puberty, endometriosis, and also for management of breast and prostate carcinoma. Thus, analogs of LHRH have various practical applications including the treatment of hormone sensitive tumors. We will review now some selected experimental and clinical findings on the agonistic and antagonistic analogs of LHRH, principally those obtained by us and our collaborators.

LHRH AGONISTS

Initially it was thought that LH releasing activity and ovulation-

inducing effects of LHRH observed in laboratory animals could be applied to the treatment of male and female infertility [15, 16]. However, the half-life of LHRH is very short [17, 18] and more potent and longer-acting analogs were considered to be necessary for clinical applications. A greater binding affinity and enzymatic resistance to proteolysis [19] became the goal for the rational design of all the LHRH analogs. Potent agonists were synthesized, but it was discovered that following chronic administration, they exert anti-fertility effects and clinical results aimed at fertility stimulation were disappointing.

Several LHRH analogues substituted in positions 6, 10 or both are much more active than LHRH (Table 1) and also possess prolonged activity [7, 20-27].

Paradoxical Antifertility Effects

Although an acute injection of superactive agonists of LHRH induces a marked and prolonged release of LH and FSH, paradoxically, chronic administration produces dramatic inhibitory effects through a process of "down regulation" of pituitary receptors for LHRH, desensitization of the pituitary gonadotropes and reduction in gonadal receptors for LH and FSH [7, 25-30]. In male animals this is manifested by a fall in the weights of testes, seminal vesicles, and prostate, a decrease in plasma testosterone levels, and a reduction in testicular LH/hCG receptors [7, 26, 30-32]. In men, a persistent suppression of Leydig cell function, manifested by a fall in serum testosterone and dihydrotestosterone levels, has been observed after chronic administration of LHRH agonists [33-35].

Chronic administration of agonistic analogs of LHRH induces paradoxical inhibitory effects on pituitary gonadotropes and gonads in female animals analogous to those reported in males. Among the effects reported in female rats are: delay in vaginal opening; reduction in ovarian and uterine weight; interference with mating and implantation; termination of gestation; and a fall in ovarian receptors for LH/hCG [7, 15, 28, 36, 37]. Antifertility effects, after administration of LHRH agonists, have also been documented in females of other species, including primate and human [15, 37, 38]. Mice are more resistant to the inhibitory effects of LH-RH agonists [39], but our recent results indicate that gonadal suppression is eventually induced [40].

Attempts are being made to utilize antifertility effects of LHRH agonists in men and women for the development of contraceptive methods [41, 42]. Continuous administration of high doses of LHRH agonists causes a suppression of circulating levels of LH (bioactive but not always radioimmunoactive) [43] and of ovarian estrogen levels [44]. The inhibition of ovarian steroidogenesis and LH forms the basis for the therapeutic use of LHRH agonists in diseases and conditions which result from inappropriate hormone levels or which can be treated by suppression of estrogens. These applications include endometriosis, polycystic ovarian disease, uterine leiomyomas, dysfunctional uterine bleeding, and hirsutism [44-47].

Therapeutic Uses

Because of potential risk of down-regulation phenomena, synthetic LHRH itself is being used for induction of ovulation, treatment of cryptorchidism and hypogonadism. Within the past three years, using automatic portable pumps designed to deliver pulses of synthetic LHRH, several investigators have demonstrated the effectiveness and safety of this mode of ovulation induction [48-50]. Treatment of cryptorchidism in prepubertal boys by intranasal administration of synthetic LHRH has also been reported [51, 52].

Female Contraception

Since the inhibition of pituitary-gonadal functions induced by chronic administration of LHRH agonists is reversible, attempts are being made to develop contraceptive methods based on the use of LHRH agonists [16, 38, 42, 53, 54]. Contraceptive action of LHRH agonists was revealed in studies on inhibition of ovulation in women when repeated administration was used. Nillius and collaborators reported that chronic subcutaneous or intranasal administration of [D-Ser(Bu^t)^6,Pro^9-NHEt]LHRH (HOE766) to regularly menstruating women could inhibit ovulation [38, 41]. More recently, Gudmundsson reported a study in which forty seven women used intranasal nafarelin for contraception over a period of six months. Ovulation was inhibited during 261 of 262 treatment months and no pregnancy occurred [55]. However, some problems may exist with this approach. Spotting, amenorrhea, unpredictable follicular maturation, effects of unopposed estradiol secreation on the endometrium as well as hypoestrogenism leading to osteoporosis [42, 54-58] must be more carefully investigated before this method could be accepted for long term contraception even with progestins as supplements.

Other approaches to female contraception based on LHRH agonists may also be linked with major problems. Although LHRH and its superactive agonists induce luteolysis in women and in monkeys if administered in mid luteal phase [59], the practical application of this finding for prevention or interception of implantation is highly limited because of the careful timing required for administration of LHRH agonists [60]. It is improbable that LHRH agonists can be used as abortifacients or interceptives in women [61, 62].

Male Contraception

It is also unlikely that LHRH agonists will be suitable as male contraceptives. Linde et al, [63] reported a fall in testosterone levels and inhibition of spermatogenesis in 8 normal men after subcutaneous treatment with [D-Trp^6,Pro^9-NHEE]LHRH for 6-10 weeks. However, 5 men became impotent and had to discontinue the treatment [63]. Replacement therapy with long acting testosterone perparations would prevent the decline in libido and forestall the hot flashes seen after LHRH agonists [64]. Bhasin et al., [65]

and Doelle and colleagues [66] noted only a partial suppression of spermatogenesis using a combined regimen of LHRH agonists with testosterone. Moreover, long-term administration of exogenous testosterone in methods intended for male contraception might lead to an increased incidence of prostate cancer and/or BPH in men [67]. The suitability of this method for male contraception is questionable.

Endometriosis

That LHRH agonists may be useful in the management of endometriosis was first shown by Meldrum et al., [68] and Lemay and Quesnel [69]. Meldrum et al., [68] treated 5 women with proven pelvic endometriosis with agonist [D-Trp^6,Pro^9-NHEt] LHRH for four weeks and achieved a "medical oophorectomy".

In our study [70], 8 menstrually cycling women with endometriosis were treated with a delayed-release preparation of [D-Trp^6]LHRH in biodegradable microcapsules for a period of 3 to 5 months. A sustained hypogonadal state, characterized by low estradiol levels was induced in all patients in 2 weeks. In all patients, estradiol levels fell to zero after the second or third injection but gonadotropins remained in the normal range. Hot flashes and severe dyspareunia were the main side effects. Resumption of normal pituitary-ovarian activity was documented in all patients 40-60 days after the last injection of microcapsules. All patients showed a clinical improvement. Other studies resulted in similar findings [71].

Polycystic Ovarian Disease

Chang et al. [44] investigated the effects of chronic administration of LHRH agonists in polycystic ovary syndrome. The concentration of estradiol declined throughout treatment. Complete suppression of ovarian androgen production may be due to the marked reduction of bioactive LH levels [43, 44].

Ayalon et al. [72] carried out a study in which 17 patients with polycystic ovarian disease (PCOD) and severe ovarian hyperstimulation syndrome (OHSS) during therapy with HMG/HCG were treated with [D-Trp^6]LHRH daily SC or in microcapsules until medical gonadectomy was attained. Under the suppressive therapy with the LHRH agonist, ovulation was induced with hFSH/HCG. In 15 of 17 patients, ovulatory cycles were obtained and 7 patients conceived. Most patients demonstrated symptoms of mild OHSS and only one patient developed severe OHSS. LHRH agonist administration for several months, to reduce ovarian androgen production and ameliorate the ovarian cystic disease process, followed by exogenous gonadotropin treatment may allow successful ovulation induction [72, 73].

In Vitro Fertilization and Embryo Transfer (IVF-ET) and Gamete Intra Fallopian Transfer (GIFT)

In IVF-ET and GIFT programs, LHRH analogs can be used to suppress

ovarian function early in the follicular phase and then an hMG followed by hCG regimen is employed to induce ovulation [74-76]. The ova may be harvested for in vitro manipulation and transfer or GIFT. The elimination of interference from endogenous gonadotropins appears to reduce the risk of ovarian hyperstimulation [74-76].

Leiomyomas

Filicori et al. [77] and Healy et al. [78] reported that the size of uterine leiomyomata could be reduced in patients by long-term treatment with LHRH agonists. Maheux and Lemay [47] employed buserelin for therapy of patients with uterine tumors. They found that this treatment resulted in tumor shrinkage, presumably because of the induction of a state of hypoestrogenism.

Coddington et al. [79] evaluated the rate at which uterine leiomyomas shrink in response to the induction of a state of hypoestrogenism by the administration of an LHRH analog. Even in patients with relatively small uterine leiomyomata, they observed at least a 50% reduction in uterine volume. Similar results were obtained by Van Leusden [80].

Perl et al. [81] used chronic administration of [D-Trp^6] LHRH to suppress the secretion of ovarian steroids for treatment of leiomyomata uteri. Ten menstruating women, presenting with a total of 20 uterine leiomyomas, were treated for three months by daily subcutaneous injections. The volume of seven uterine leiomyomas diminished by more than 80%, while 8 leiomyomas decreased partially. After an initial reduction, 5 leiomyomas re-enlarged during the last month of treatment. The agonist was well tolerated and few side effects were observed. It was concluded that therapy with LHRH agonists offers a good alternative in the management of some uterine leiomyomas [81] Similar findings were obtained by George et al. [82]. These results suggest that LHRH agonist-induced hypoestrogenism may be a useful method for management of uterine leiomyomata, as a primary therapy or as an adjunct to leiomyomectomy.

Ovarian Cancer

Gonadotropins might increase the incidence of cancers of the ovary in animals and humans [31]. The effects of chronic administration of agonists of LHRH are being investigated in murine models and in nude mice bearing transplanted human ovarian epithelial tumors. Suppression of the secretion of gonadotropins produced by LHRH agonists appears to inhibit the growth of ovarian tumors [31, 42]. Parmar et al. [83] reported that chronic treatment with [D-Trp^6]LHRH microcapsules induced the regression of an inoperable, bilateral, serous cystadenocarcinoma of the ovary in a 78 year old woman. More recently, these findings were confirmed and extended in a trial of 39 women with epithelial ovarian cancer [84]. Eleven patients showed remission or stabilization of disease.

Prostate Cancer

A marked inhibition of pituitary and gonadal function that occurs after chronic administration of agonists of LHRH, made possible a new approach for the treatment of sex hormone-dependent tumors [30, 31, 42]. In our initial studies, we have shown that chronic administration of [D-Trp^6]LHRH suppressed tumor growth in rats with Dunning R3327H prostate cancers and reduced serum levels of testosterone [85]. Subsequent clinical trials documented marked improvement in patients with stage C or D prostate carcinoma after treatment with [D-Trp^6]LHRH, buserelin, leuprolide or zoladex [86-95]. Initially, superagonists of LHRH were given daily by the subcutaneous or intranasal route [86-95]. Subsequently, we developed a long-acting delivery system for [D-Trp^6]LHRH in microcapsules of poly (DL-lactide-co-glycolide) designed to release a controlled dose of the peptide over a 30-day period [96, 97].

Microcapsules of [D-Trp^6]LHRH that can be injected once a month make the treatment of patients with prostate carcinoma and other neoplasms more convenient. The efficacy of the slow-release formulation of [D-Trp^6]LHRH microcapsules in the treatment of advanced prostatic carcinoma was demonstrated in animal experiments and in clinical trials [96, 98, 99]. In a trial with microcapsules in men with prostate cancer carried out with Parmar, Lightman and Phillips, we obtained 87% objective response in the microcapsule treated group vs. 81% for total orchidectomy [99].

The existing results warrant continuation of clinical trials with LHRH agonists for the treatment of advanced prostate carcinoma. However, it is possible that the therapeutic response could be improved by combining LHRH agonists with other compounds including peptides such as somatostatin analogs and various chemotherapeutic agents [100-105].

The duration of remission in patients with prostate cancer may be limited as hormonal manipulations do not prevent the ultimate growth of hormone-independent cells [106, 107]. Combination of hormonal therapy with chemotherapy could forestall this phenomenon and prolong survival [107]. The effect of combining hormonal treatment consisting of microcapsules of [D-Trp^6]LHRH with various chemotherapeutic agents has been investigated in the Dunning prostate cancer model. The combination of cyclophosphamide (Cytoxan) with the microcapsules was much more effective than the single agents in reducing tumor growth [103]. Novantrone (Mitoxantrone), an anthracenedione similar to adriamycin, but less toxic, was also used in combination with microcapsules of [D-Trp^6]LHRH [104]. Again, the combination with Novantrone led to a better inhibition of prostate cancer than [D-Trp^6]LHRH microcapsules alone and in fact, arrested tumor growth [104]. Pathological examination of the tumors showed that the combination therapy inhibited the growth of tumor cells and that these cells were replaced by connective tissue [108].

Prolactin could be involved in prostate cancer as a cofactor [101, 102, 109]. Somatostatin analogs inhibit prolactin and

growth hormone secretion and have direct antiproliferative effects on cells [101, 102, 105]. The reduction in prolactin levels produced by the administration of somatostatin analogs, combined with the decrease in serum testosterone resulting from chronic treatment with LHRH agonists, may inhibit growth of prostate tumors better than LHRH agonists alone [101, 102, 105].

The fall in GH levels induced by somatostatin analogs could, through mechanisms involving endogenous growth factors, be even more important for the inhibition of tumor growth than the reduction in prolactin [105]. GH stimulates local production of insulin-like growth factor 1(IGF-1) [105]. IGF polypeptides (also called somatomedins) and various growth factors including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF) and others, appear to be involved in the proliferation of both normal and neoplastic cells or phenotypic transformation of cells [105]. In cancer cell lines, somatostatin can reverse the stimulatory effect of EGF on the phosphorylation of the tyrosine kinase portion of the EGF receptor [100, 110]. Some of our superactive analogs of somatostatin such as RC-160 and RC-121 exhibit even higher activity on dephosphorylation of EGF receptor [110].

Thus somatostatin analogs might inhibit prostate cancers by reducing release of GH and prolactin and interfering with the action, signal transmission or secretion of endogenous growth factors [105]. Modern superactive octapeptide analogs of somatostatin, including RC-121, significantly decreased the weight and volume of Dunning R3327H prostate cancers and, when given in combination with once-a-month [D-Trp^6]LHRH microcapsules, potentiated the effects of the latter [105]. Microcapsules of RC-160 designed for a controlled release of this analog for 30 days also inhibited the growth of Dunning prostate tumors when given alone. Combination of microcapsules of [D-Trp^6]LHRH with microcapsules of RC-160 resulted in a synergistic potentiation of the inhibition of prostate tumors [105]. Serum GH and PRL levels in treated groups were decreased. Somatomedin-C levels in extracted serum of rats treated for 84 days with somatostatin analog RC-160, alone or in combination with [D-Trp^6]LHRH, were significantly reduced as compared to control. Histopathological evaluation of prostate cancers in the group of rats which received the combination treatment showed an inhibitory effect on tumors nearly equivalent to that induced by the combination of novantrone and [D-Trp^6]LHRH [111].

LHRH analogs have also been used for the treatment of benign prostate hyperplasia (BPH) and shown to cause a regression of the prostate [112, 113].

Breast Cancer

About 30% of breast cancers in women are estrogen dependent [114]. Various experimental studies suggest that analogs of LHRH might be useful for treatment of estrogen-dependent breast cancer [30, 31, 115, 116]. In rats bearing the MT/W9A mammary adenocarcinoma, chronic administration of [D-Trp^6]LHRH

injected daily or once a month as microcapsules, or the antagonist [Ac-D-p-Cl- Phe^{1,2}D-Trp^{3},D-Arg^{6},D-Ala^{10}]LHRH decreased tumor weight and volume [115]. Regression of mammary tumors in rats and mice in response to agonists and antagonists of LHRH suggested that these compounds should be considered for a new hormonal therapy for breast cancer in women.

In clinical trials carried out so far, regression of tumor mass and disappearance of metastases in premenopausal and postmenopausal women with breast cancer treated with [D-Trp^{6}] LHRH, buserelin or leuprolide have been described [30, 31, 117-122]. Some direct antitumor effects of LHRH analogs on mammary carcinomas are also possible since several groups, including ours, found LHRH receptors in human breast cancers [123, 124]. The use of microcapsules or depot implants of LHRH agonists makes the treatment of breast cancer with LHRH agonists more convenient and ensures patient compliance. It is also possible that treatment with LHRH agonists can be combined with chemotherapy or with peptides that inhibit prolactin release such as somatostatin analogs or prolactin release-inhibiting factor (PIF) [101]. Since prolactin and GH may act as promoters in the development or growth of mammary tumors, the reduction in prolactin and GH levels induced by somatostatin analogs and interference with growth factors, may potentiate inhibition of mammary tumors obtained with LHRH alone [101]. In rats bearing estrogen- and prolactin-dependent MT/W9A mammary adenocarcinoma, daily injections of somatostatin analogs inhibited the growth of this tumor [101]. Microcapsules of somatostatin analog RC-160 also significantly inhibited the growth of the mammary tumor. A significant synergism between the LHRH agonist and the somatostatin analog in the inhibition of tumor growth was demonstrated when both peptides were given together. At present, somatostatin analogs are being tried alone or as adjuncts together with agonistic analogs of LHRH in the treatment of breast cancer in women, depending upon the status of receptors [101].

Pancreatic Cancer

Our experimental studies [31, 101, 125-127] and those of others [128-132] are consistent with the view that the exocrine pancreatic carcinomas are sensitive to G.I. hormones, sex steroids and growth factors. Some clinical observations support the hyppthesis that pancreatic cancers might be, in part, sex-hormone dependent [133, 134]. This subject is discussed in detail in the accompanying article [135]. Using animal models of transplanted pancreatic acinar and ductal cancers, we investigated the effect of analogs of LHRH and somatostatin on the growth of these tumors [125]. [D-Trp^{6}]LHRH, given daily or injected once a month as microcapsules, significantly decreased tumor weight and volume in both models and suppressed serum levels of testosterone [125]. Some somatostatin analogs also inhibited tumor growth [125]. Similar inhibition of tumor growth by [D-Trp^{6}]LHRH and somatostatin analog was obtained in female golden hamsters in which ductal pancreatic cancers were induced by administration of

nitrosamine [126, 127]. This suggests that pancreatic cancers may be sex hormone-sensitive. [D-Trp^6]LHRH may decrease the growth of pancreatic carcinomas by eliminating the effects of sex steroids. On the basis of these experimental observations that administration of [D-Trp^6]LHRH inhibits the growth of the ductal and acinar pancreatic cancers, this analog was tried clinically in a small number of patients with inoperable pancreatic cancer [134]. The trial was carried out in collaboration with Gonzalez-Barcena in Mexico City. Some patients showed clinical improvement, reduction in tumor mass and increased survival rate.

Gastrin, cholecystokinin and secretin produce hyperplasia and hypertrophy of the exocrine pancreas and might influence the growth of the malignant cells of the pancreas as well [31, 101, 132].

In Syrian hamsters, modern somatostatin analogs, such as D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH_2 (RC-160), inhibited the growth of ductal pancreatic tumors [126, 127]. These findings suggest that pancreatic adenocarcinoma may be sensitive to both gastrointestinal and sex hormones. The growth of MIA PaCa-2 human pancreatic cell line in nude mice was also inhibited by RC-160 and [D-Trp^6]LHRH. The combination of both types of analogs appears to exert the greatest inhibitory effect on pancreatic cancers. Somatostatin analogs reduce the growth of pancreatic ductal and acinar cancers, probably by inhibiting the release and/or stimulatory action of gastrointestinal hormones on tumor cells [31, 101] and by interfering with the action and secretion of growth factors [127]. The stimulatory effect of EGF on the growth of human pancreatic MIA PaCa-2 cells in vitro can be abolished by somatostatin-14 [136]. Somatostatin analogs and [D-Trp^6]LHRH should be considered for the development of a new hormonal therapy for cancer of the pancreas.

LHRH ANTAGONISTS

While repeated chronic administration of LHRH agonists is required to inhibit LH and FSH release and reduce the levels of sex steroids, similar effects can be obtained with a single administration of LHRH antagonist [31, 42, 102, 137].

Antagonistic analogs of LHRH were developed for contraception [137]. Modern antagonists possess modifications in positions 1, 2, 3, 6, 10 and others [138]. These antagonists act on the same receptor sites as LHRH and cause an immediate inhibition of the release of gonadotropins and sex steroids. Since 1972, hundreds of LHRH antagonists have been synthesized and assayed in animals. Some of the more potent antagonists were also tested in human beings [137-139]. [D-Phe^2,D-Trp^3,D-Phe^6]LHRH was the first inhibitory analog found to be active in human beings [140]. The development of LHRH antagonists has been reviewed previously [137,138]. Insertion of D-arginine in the position 6 of LHRH antagonists increases antiovulatory activity [141]. [Ac-D-p-Cl-Phe^{1,2},D-Trp^3,D-Arg^6,D-Ala^{10}]LHRH (ORG 30276) exhibited antiovulatory activity at a dose of 1-3μg

[141]. High in vivo potency of D-Arg^6 analogs can be further increased by the introduction of the very hydrophobic residue, [D-Nal(2)], at position 1[138-139].

Various studies established that LHRH antagonists can decrease serum gonadotropins, and block ovulation in rats, hamsters and rabbits [42, 137, 142]. In male rats, chronic administration of LHRH antagonists inhibits LH, FSH and testosterone levels and suppresses spermatogenesis [143, 144]. In a series of extensive studies Asch et al, [145, 146] demonstrated inhibitory effects of modern LHRH antagonists on gonadotropin production and ovulation in monkeys. [N-Ac-D-p-Cl-Phe^{1,2} D-Trp^3,D-Arg^6,D-Ala^{10}]LHRH has also been shown to depress serum levels of LH, FSH and testosterone in normal male rhesus monkeys [147]. Several LHRH antagonists have been tested in men and women and demonstrated to be active and powerful enough for clinical use [137, 148, 149].

Clinical Trials with Antagonists of LHRH

In 1977 we demonstrated that large doses of [D-Phe^2,D-Trp^3, D-Phe^6]LH-RH diminished the gonadotropin response to LHRH in normal men (140). In normal ovulatory women, this antagonist disrupted the menstrual cycle and ovulation did not occur [148]. In oophorectomized or postmenopausal women, we found that the administration of [D-Phe^2,D-Trp^3,D-Phe^6]LHRH decreased LH and FSH levels up to 24 hours after injection [150].

In another study a still more powerful antagonist, [N-Ac-D-p-Cl-Phe^{1,2},D-Trp^3,D-Phe^6,D-Ala^{10}]LHRH, administered on day 12 in a dose of 5mg, blocked ovulation in 6 out of 9 normal women [137]. No breakthrough bleeding occurred. [N-Ac-D-p-Cl-Phe^{1,2},D-Trp^3,D-Phe^6,D-Ala^{10}]LHRH was then tested in smaller doses in 10 normally menstruating ovulatory women [149]. Intramuscular administration of the antagonist in doses of 2mg on day 12 of the menstrual cycle inhibited the midcycle surge of LH and FSH and ovulation in 6 out of 10 women. Serum progesterone levels and urinary pregnanediol values in these women were consistently low, and corresponded to anovulatory cycles. These women menstruated 12 to 26 days after injection of the antagonist. Two patients in whom the analog did not abolish the LH mid-cycle surge showed a short luteal phase and premature menstruation. An early decline in progesterone levels suggested that luteolysis took place [149].

Other groups also reported clinical studies on LHRH antagonists [151]. A powerful LHRH antagonist, [Ac-dehydro-Pro^1,D-p-F-Phe^2,D-Trp^{3,6}]LHRH, administered intravenously in doses of 80μg/kg, (about 5mg/patient), lowered serum levels of LH and FSH within 5 hours in hypergonadotropic postmenopausal women, and decreased the pulses of both gonadotropins. It was also shown that single subcutaneous injection of this antagonist suppresses serum gonadotropin and testosterone levels in normal men without any side effects [152]. Similarly, [N-Ac-D-Nal(2)^1, D-pCl-Phe^2,D-Trp^3,D-hArg (Et)$_2$6,D-Ala^{10}]LHRH (Detirelix;RS-68439) lowered serum LH, FSH and testosterone and caused no systemic side

effects after s.c. injection in normal men in doses of 5-20mg [153].

However, side effects have been observed with most LHRH antagonists with D-Arg in the 6 position. Thus, several such LHRH antagonists in doses of 1.25mg/kg (s.c.) caused a transient allergic reaction in rats (swollen tail, legs, face, ears), suggesting a local and general edema. This reaction is probably due to histamine liberation [154-157]. This phenomenon is species specific: mice, rabbits, dogs or rhesus monkeys do not show this phenomenon [154-157].

In preliminary human tolerance studies in Europe in women, an erythema upon the skin at the side of the subcutaneous injection was observed in some cases after administration of ORG 30276 in doses of 9.5mg[144]. This reaction could be due to histamine liberation. These side effects delayed clinical use of some LHRH antagonists in humans.

Recent LHRH Antagonists

In order to eliminante the undesirable edematogenic effect of the LHRH antagonists containing basic D-amino acids at position 6, exemplified by [Ac-DpCl-Phe^{1,2},D-Trp^{3},D-Arg^{6},D-Ala^{10}] LHRH, new analogs with D-ureidoalkyl amino acids such as D-Cit, D-Hci, at position 6 were synthesized in our laboratory and tested in several in vitro and in vivo systems [158]. HPLC analysis revealed that the overall hydrophobicity of the new D-Cit/D-Hci^{6} analogs was similar to that of the basic D-Arg^{6} antagonists. In vitro, most of the new analogs completely inhibited LHRH-mediated LH release in perfused rat pituitary cell systems at an antagonist to LHRH ratio of 5:1. In vivo, the most active peptides, e.g. [Ac-D-Nal(2)^{1},D-pCl-Phe^{2},D-Trp^{3},D-Cit^{6},D-Ala^{10}]LHRH caused 100% inhibition of ovulation in cycling rats in doses of 3µg and suppressed the LH level in ovariectomized female rats for 47 hours when administered at levels of 25µg[158]. Characteristically, these peptides did not exert any edematogenic effects even at a dose of 1.5mg/kg. These properties of the D-Cit/D-Hci^{6} antagonists may make them useful clinically.

Other groups have also reported different structural modifications which diminish anaphylactoid toxicity, in particular the histamine releasing activity of the D-Arg^{6} antagonists [159, 160]. In the first approach, Arg was transposed from position 6 to 5 and a hydrophobic D-residue was introduced into position 6 to yield analogues such as [Ac-D-Nal(2)^{1},D-pCl-Phe^{2},D-Pal(3)^{3}, Arg^{5},D-Abu(AA)^{6},D-Ala^{10}]LHRH [159]. In the other approach, moderately basic acylated L-Lys and D-Lys were introduced into positions 5 and 6, respectively, and Arg^{8} was replaced with alkylated Lys or Orn to obtain peptides such as [Ac-D-Nal(2)^{1}, D-pCl-Phe^{2},D-Pal(3)^{3},Lys(Nic)^{5},D-Lys(Nic)^{6},Lys(Ipr)^{8},D-Ala ^{10}]LH-RH [160]. Both the peptides showed diminished histamine releasing potency.

In view of successful clinical use of LHRH agonists in the treatment of prostate cancer [86-99], breast cancer [117-122], leiomyomas [77-81], endometriosis [68-71] and precocious puberty

[161, 162] as well as experimental efficacy of LHRH antagonists in animal models of prostate cancer [163] and breast cancer [115], the development of LHRH antagonists should continue. The advantage of the antagonists would be based on the fact that they inhibit LH, FSH and sex steroids secretion from the start of the administration. The use of antagonistic analogs of LHRH for the treatment of cancer and other conditions would avoid the transient stimulation of the release of gonadotropins and sex steroids which occurs initially in response to LHRH agonists, thus preventing the temporary clinical "flare-up" of the disease. In addition to contraception, LHRH antagonists could find applications for the treatment of homone dependent cancers as well as conditions such as endometriosis, leiomyomas and precocious puberty, where the depression of pituitary-gonadal function is desirable. The development of long-acting delivery systems of the LHRH antagonists for once a month or even less frequent administration, will be essential for a practical contraceptive method and other clinical uses. These formulations may be similar to those developed for LHRH agonists [96, 97, 164].

GONADAL PROTECTION AGAINST CHEMOTHERAPY AND RADIATION

Actively dividing cells are more sensitive than resting cells to some chemotherapeutic agents and to radiation [165-167]. Therefore, we administered the agonist [D-Trp^{6}]LHRH or the powerful LHRH antagonist [Ac-D-pCl-Phe^{1,2},D-Trp^{3},D-Arg^{6}, D-Ala^{10}]LHRH to rats or subhuman primates for 2-3 months before radiation or chemotherapy, in order to suppress the pituitary-gonadal function and maintain inactive testes [165-167]. The baboon pretreated with [D-Trp^{6}]LHRH before 4 months of therapy with cytoxan showed a recovery of spermatogenesis, but the control animal given only cytoxan showed azoospermia [166]. Similar results were obtained in rats pretreated with [D-Trp^{6}]LHRH or the antagonist before administration of procarbazine (matulane) [167]. When x-radiation was used to inflict gonadal damage, rats pretreated with LHRH antagonist showed a complete recovery of testicular weights and spermatogenesis 3 months after 415 rads and partial recovery after 622 rads; LH and FSH levels returned to normal in both of these groups [165].

These results indicate that radiation and chemotherapy damage the testes temporarily inhibited by LH-RH analogs to a lesser extent than the unsuppressed gonads, and the recovery of reproductive function is accelerated in animals treated with the analogs [165-167]. Our findings have clinical implications and suggest the merit of continued exploration of LHRH analogs as gonadal protectors against chemotherapy and radiation.

CONCLUSIONS

Many clinical approaches are being developed based on analogs of LHRH. The phenomena of pituitary desensitization and inhibition of sex steroid levels by LHRH agonists are being used for treat-

ment of precocious puberty, endometriosis and benign prostate hyperplasia. LHRH agonists are also being tried in contraception and employed in polycystic ovarian disease and IVF-ET or GIFT. Inhibition of the pituitary-gonadal axis forms the basis for oncological applications of LHRH agonists. Various endocrine-dependent or hormone-sensitive tumors can be treated with LHRH analogs. The use of delayed delivery systems based on microcapsules or implants makes the treatment more practical and efficacious. A successful utilization of agonistic analogs of LHRH for the treatment of androgen-dependent prostate cancer has been documented in hundreds of patients. Agonists or antagonists of LHRH might be also beneficial for treatment of breast cancer in premenopausal and postmenopausal women. The development of LHRH antagonists free of edematogenic effects is continuing. Work is in progress on the application of LHRH analogs for the treatment of ovarian cancer, neoplasms of the female genital tract such as leiomyomas, endometrial carcinoma and for protection against gonadal damage during chemotherapy and radiation. Methods based on the analogs of LHRH or somatostatin might supplement or in some cases replace conventional procedures for the treatment of hormone-sensitive cancers.

It is gratifying to see that the discovery of LHRH has led to so many practical clinical uses. The list of these applications might increase further, particularly for the antagonists. Additional new classes of antitumor drugs might be developed based on LHRH.

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2
CHEMISTRY OF GnRH ANALOGUES

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INTRODUCTION

The mammalian gonadotrophin releasing hormone structure was first published by Schally et al., in 1971 from porcine hypothalamus [1] and soon afterwards by Guillemin et al., from ovine hypothalamus [2]. Due to the enormous clinical potential of this peptide a large number of agonist and antagonist analogues have been synthesised in the last seventeen years. The structure-activity relationships of all these analogues cannot be summarised in the limited space available here. The main purpose of this review is, therefore, to identify and discuss only those changes which have been incorporated into the agonist and antagonist analogues which are either in the market or in advanced stages of development. Detailed structure-activity studies of agonist and antagonist analogues, and their clinical and biological studies have been reviewed elsewhere [3-7].

Until 1980 GnRH variants from other species had not been isolated and sequenced. Only recently the structures of chicken, salmon and lamprey GnRH have been published and these are shown in Table 1 along with the structure of the porcine and ovine GnRH [8]. Additional GnRH-like peptides have also been found in alligator, skink and lizard. The alligator and skink GnRH were similar to the chicken GnRH but of the three GnRH sequences identified in lizard one was similar to salmon and the other two were novel [8].

Due to the absence of structural data on the other naturally occurring GnRH-like peptides before 1980 all of the early work on the agonist analogues was based on the original porcine/ovine sequence. Some of the new information is however presently being used in the design of antagonist analogues.

STRUCTURE-ACTIVITY RELATIONSHIPS OF THE AGONIST ANALOGUES

The agonist analogues of GnRH which are presently being marketed, or are in advanced stages of clinical trials, are listed in Table 2. Of the analogues listed in the table goserelin,

Table 1. Structures of the GnRH molecules isolated from various species.

Origin	Sequence									
	1	2	3	4	5	6	7	8	9	10
Porcine,	Glu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH_2
Ovine										
Chicken I	----------									Gln----------
Chicken II	----------							His	-----	Trp-Tyr----------
Salmon	----------									Trp-Leu----------
Lamprey	--------		Trp	------		Leu	Glu	Trp	Lys	----------

Table 2. Structures of GnRH analogues undergoing further evaluation.

Agonist Structure	Name (Company)
[D-Ser(Bu^t)^6,Azgly^{10}]-LHRH	'Zoladex' (ICI)
[D-Ser(Bu^t)^6,des-Gly-NH_2 ^{10}]LHRH(1-9)NHEt	Buserelin (Hoechst)
[D-Trp^6]-LHRH	Tryptorelin (Debiopharm) (Decapeptyl)
[des-Gly-NH_2 ^{10}]-LHRH(1-9)-NHEt	Fertirelin (Takeda)
[D-His(Bzl)^6,des-Gly-NH_2 ^{10}]-LHRH(1-9)NHEt	Histrelin (Ortho)
[D-Leu^6,des-Gly-NH_2 ^{10}]-LHRH(1-9)NHEt	Leuprolide (Abbott)
[D-Trp^6,MeLeu^7,des-Gly-NH_2 ^{10}]-LHRH(1-9)NHEt	Lutrelin (Wyeth)
[D-Nal(2)^6]-LHRH	Nafarelin (Syntex)

buserelin, tryptorelin and leuprorelin are already being marketed for the treatment of prostate cancer and histrelin and nafarelin are in advanced stages of clinical trials. These six analogues have either one or two changes from the parent peptide. In tryptorelin and nafarelin only the Gly^6 residue has been replaced by a D-amino-acid residue but in the remaining four the C-terminal glycine residue has also been modified. Goserelin has an azaglycine residue in position 10 but buserelin, histrelin and leuprorelin have an ethylamide group in place of the C-terminal Gly-NH_2. The significances of these position 6 and 10 changes are summarized below.

Replacement of the C-terminal Gly-NH_2 by an ethylamide group

Elimination of the C-terminal glycine residue gave a nonapeptide with about 10% biological activity (in vitro LH release). Further reduction in the chain length from the C-terminus led to inactive compounds (<0.01% GnRH). When the Gly-NH_2 was replaced with various linear, branched or cyclic alkylamines, e.g. a C-terminal ethylamide, propylamide or isopropylamide, some of the resulting analogues, were 2-3 times more potent than GnRH in releasing LH in pituitary cell cultures [9,10]. Other analogues with C-terminal methyl, dimethyl or butylamide groups were somewhat less potent. These early results formed the basis of all the agonists containing a C-terminal ethylamide group, e.g. buserelin, histrelin and leuprorelin.

Replacement of the C-terminal Gly-NH_2 by an α-aza-amino-acid

α-Aza-amino-acid replacements in peptides are likely to induce conformational changes which may lead to increased receptor affinity and at the same time may also stabilise the peptide bonds to enzymic degradation. Detailed conformational studies on α-aza-peptides have not yet been reported but the enzymic stability of model azapeptides to various enzymes has been investigated [11]. The aza-residue was found to stabilise the peptide bonds (formed by the amino and the carboxyl groups of these residues) to aminopeptidase, carboxypeptidase and thermolysin. The effects on stability were comparable to those of the D-amino-acid residues.

The analogues containing a single aza-residue, e.g. [$Azgly^{10}$]-GnRH and [$Azala^{10}$]-GnRH, were as potent as GnRH in inducing ovulation in androgen-sterilised, constant oestrous rats [12, 13]. In contrast, [Ala^{10}]-GnRH and [Pro^{10}]-GnRH had earlier been shown to be much less potent (<5% GnRH) [10]. The 25-fold difference between the activities of [$Azala^{10}$]-GnRH and [Ala^{10}]-GnRH probably reflects the advantages of the conformational change induced by an aza-alanine residue.

Replacement of Gly^6 with various L and D-amino-acid residues

Substitution of another L-amino-acid residue in position 6, e.g. Ala, Ile, Val, Pro, or some D-amino-acid residues, e.g. D-Val,

D-Pro, resulted in much less potent analogues (0.1 to 32% GnRH). In contrast replacement with a number of other D-amino-acid residues, e.g. D-Ala, D-Leu, D-Phe, D-Trp, D-Nal(2), D-Arg, D-hArg(Et_2), D-Ser(Bu^t), D-Tmp, D-Dmb, led to analogues which were 30 to 200-fold more potent than GnRH [3,14-19]. Because of this enormous increase in potency the D-amino-acid residues in position 6 play an essential role in all of the agonists presently in development.

Replacements in position 6 and 10

Various combinations of substitutions in positions 6 and 10 have been attempted in order to improve the potency even further but this approach has not been universally successful. The C-terminal ethylamide analogues of [D-Ala^6]-GnRH, [D-Leu^6]-GnRH (Leuprolide; leuprorelin) and [D-$Ser(Bu^t)^6$]-GnRH (buserelin) were more potent than the corresponding Gly-NH_2 ^{10} analogues, but [D-$Nal(2)^6$]-GnRH and [D-$Nal(2)^6$,N-$MeLeu^7$]-GnRH were more potent than the corresponding ethylamide analogues. In the position 10 aza-amino-acid series of analogues [D-Phe^6, $Azgly^{10}$]-, [D-$Tyr(me)^6$,$Azgly^{10}$]-, [D-$Ser(Bu^t)^6$, $Azgly^{10}$]- (Zoladex; goserelin), [D-$Nal(2)^6$,$Azgly^{10}$]- and [D-Dmb^6 $Azgly^{10}$]-GnRH were extremely potent [20-22].

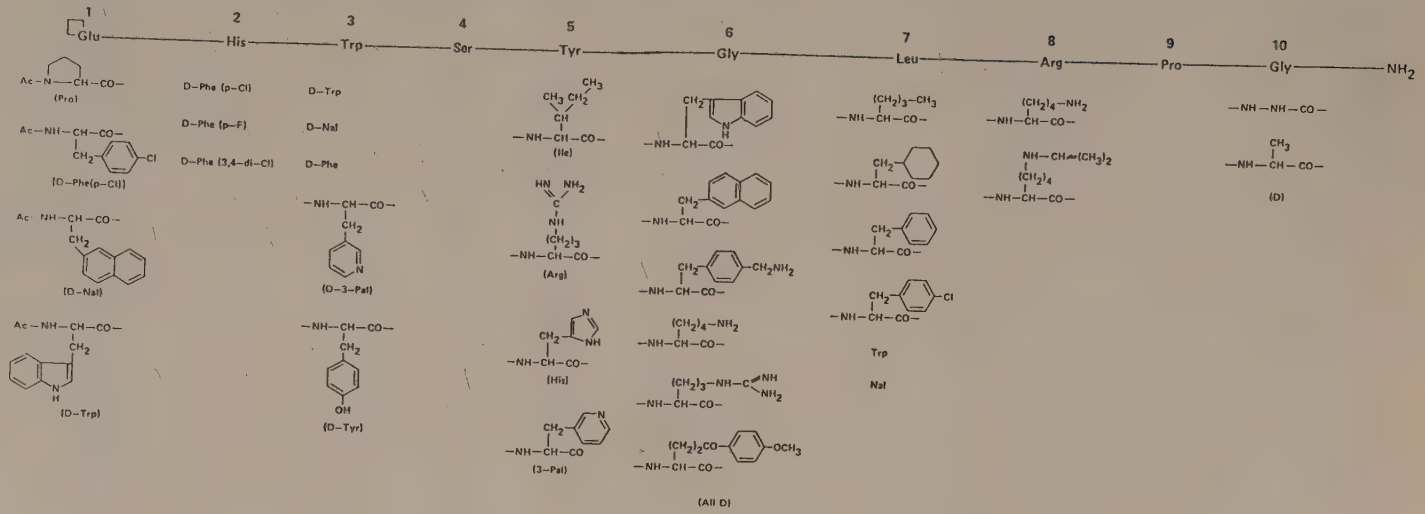
STRUCTURE-ACTIVITY RELATIONSHIPS OF GnRH ANTAGONIST ANALOGUES

First antagonists of GnRH, discovered within a year of the discovery of GnRH itself, were obtained either by eliminating the whole of the histidine residue or just its side-chain [4, 23, 24]. Although [des-His^2]-and [Gly^2]-GnRH were weak antagonists (active only in in vitro test systems) their discovery pointed to the key to antagonism, and led to the synthesis of a large number of position 2 modified analogues. The majority of these, e.g. Ser, Thr, Gln, Arg, Leu, Phe, Tyr, Trp, D-Arg, D-Leu and D-Val^2 analogues, were only weak agonists. Only [D-Phe^2]- and [D-Trp^2]-GnRH were more potent antagonists than [des-His^2]-GnRH.

Since changes in positions 6 and 10 had resulted in significant enhancement in potency in the agonist series of analogues these changes were also made in the antagonist series of analogues along with position 2 changes. All the des-His^2 and D-Phe^2 analogues with D-amino-acid residues in position 6, e.g. [des-His^2,D-Ala^6]-, [des-His^2,D-Lys^6]-, [des-His^2, D-Orn^6]-, [D-Phe^2,D-Ala^6]-, [D-Phe^2,D-Phe^6]-, and [D-Phe^2,D-Trp^6]-GnRH, were more potent antagonists of GnRH than the parent [des-His^2]- or [D-Phe^2]-GnRH [25,26].

The C-terminal ethylamide substitution was not successful in the antagonists. Most of the ethylamide analogues of position 2 or positions 2 and 6 modified compounds, e.g. [des-His^2, D-Lys^6, des-Gly-NH_2^{10}]-GnRH(1-9)NHEt, [D-Phe^2,D-Ala^6, des-Gly-NH_2^{10}]-GnRH(1-9)NHEt, were much less potent than the corresponding Gly^{10} decapeptides in inhibiting ovulation in rats

Table 3 Amino-acid Substituents Leading to potent LHRH Antagonists



[25,27]. In contrast to the results with the C-terminal ethylamide analogues the results with an azaglycinamide substitution in position 10 were more promising. [des-His^2, $Azgly^{10}$]- and [des-His^2,D-Phe^6, $Azgly^{10}$]-GnRH blocked ovulation completely at a dose of 250 µg/rat and partially at 62.5 µg/rat. [D-$Phe^{2,6}$,$Azgly^{10}$]-GnRH blocked ovulation completely at a dose of 15 µg/rat and was about 10-fold more poent than [D-$Phe^{2,6}$]-GnRH [20,28]. Similar enhancement in antagonist potency was also observed in a number of other analogues with multiple substitutions [4].

Further improvement in potency was then sought by modifications in various other positions. This led to potent GnRH antagonists which had 5-8 of the original amino-acids replaced by unnatural amino-acid residues. The preferred amino-acid residues in each position of GnRH (for high antagonists potency) are summarised in Table 3 and some of the more potent antagonists incorporating these changes are listed in Table 4 [29-34]. All of these antagonists inhibited ovulation in constant cycling rats at doses below 10 µg/kg.

TABLE 4: Potent antagonists of LHRH

LHRH	Glu—His—Trp—Ser—Tyr—Gly—Leu—Arg—Pro—Gly—NH_2
ORG 30093	Ac-D-Phe(Cl)-D-Phe(Cl)-D-Trp—D-Phe—D-Ala—
ORG 30276	Ac-D-Phe(Cl)-D-Phe(Cl)-D-Trp—D-Arg—D-Ala—
ORF 18260	Ac-D-Nal-D-Phe(F)-D-Trp—D-Arg—
RS 68439	Ac-D-Nal-D-Phe(Cl)-D-Trp—D-hArg(Et_2)—D-Ala—
	Ac-D-Nal-D-Phe(Cl)-D-3-Pal—D-Arg—D-Ala—
	Ac-D-Nal-D-Phe(Cl)-D-3-Pal—D-Arg—Trp—D-Ala—
	Ac-D-Nal-D-Phe(Cl)-D-3-Pal—Arg—D-3-Pal—D-Ala—

Table 5: Antiovulatory and histamine releasing activities of some potent GnRH antagonists

Compound	Anti-ovulatory activity (ED_{100} µg/rat)	*In vitro* histamine release (ED_{50} µg/ml)
[Ac-D-Nal(2)^1, D-Phe(p-Cl)^2, D-Trp^3, D-Arg^6, D-Ala^{10}]-LHRH	1.0	0.1
[Ac-D-Nal(2)^1, α-Me-D-Phe(p-Cl)^2, D-Trp^3, Arg^5, D-Tyr^6, D-Ala^{10}]-LHRH	2.5	3.7
[Ac-D-Nal(2)^1, D-Phe(p-Cl)^2, D-3-Pal^3, Arg^5, D-Glu(AA)^6, D-Ala^{10}]-LHRH	1.5	1.6
[Ac-D-Nal(2)^1, D-Phe(p-Cl)^2, D-3-Pal^3, Arg^5, D-3-Pal^6, D-Ala^{10}]-LHRH	1.0	2.9
[Ac-D-Nal(2)^1, D-Phe^{2,3}, D-Lys(x)^6, Phe^7, D-Ala^{10}]-LHRH		
X = H	3.0	0.37
- cyclopentyl	3.0	0.24
- cyclohexyl	3.0	0.32
[Ac-D-Nal(2)^1, D-Phe^{2,3}, D-Arg^6, Phe^7, Lys(x)^8, D-Ala^{10}]-LHRH		
X = H	>3	0.38
- neopentyl	>3	4.68
[Ac-D-Nal(2)^1, D-Phe(p-Cl)^2, D-3-Pal^3, (5), (6), (8), D-Ala^{10}]-LHRH		
(5) Nic – Lys; (6) Nic – D-Lys; (8) iPr – Lys	1.0	>300
(5) Nic – Lys; (6) Nic – D-Lys; (8) iPr – Orn	1.0	206
(5) Pic – Lys; (6) Pic – D-Lys; (8) iPr – Lys	0.5	93

An examination of the types of amino-acid residues needed for high antagonist potency and also the structures of the potent antagonists shows that the overall hydrophobicity and basicity of the original GnRH sequence are significantly increased. This combination of increased hydrophobicity and basicity has led to a problem due to histamine release induced by most of the potent antagonists. This problem has previously been associated with a number of other peptides, e.g. substance P, which contain hydrophobic and basic residues [35]. Attempts have recently been made to synthesise analogues lacking in histamine releasing activity and these can broadly be categorised into three types. The first approach involved switching the residues between positions 5 and 6. The second and third approaches were based on reducing the overall hydrophobicity and shielding the side-chain basic groups. These were achieved by replacing the D-Trp^3 with D-Pal^3 and also by incorporating various substituents, e.g. isopropyl, nicotinyl or picolyl, on the side-chain amino groups in positions 5, 6 and 8. Examples of some of these modified analogues are shown in table 5 along with their antiovulatory and in vitro histamine releasing activities [36-38]. In comparison to the parent peptide, [Ac-D-Nal(2)^1,D-pCl-Phe^2,D-Trp^3,D-Arg^6, D-Ala^{10}]-GnRH, the analogues containing an Arg residue in position 5 instead of a D-Arg residue in position 6 were 16-37 times less potent in releasing histamine. The Arg^5 analogues were only marginally less potent as antiovulatory agents. Incorporation of cyclopentyl, cyclohexyl and some other groups onto the side-chain amino group of Lys^6 did not show any improvement in the histamine releasing activity. An analogue with a neopentyl group in position 8, [Ac-D-Nal(2)^1,D-Phe^{2,3},D-Arg^6, Phe^7,Lys(neopentyl)^8,D-Ala^{10}]-GnRH, was about 10-15 times less potent in releasing histamine than the corresponding Lys^8 analogue but both of these analogues were also less potent as antiovulatory agents (ED_{100} >3 μg/rat). Analogues with side-chain modifications in positions 5, 6 and 8 gave the most promising results. A number of such analogues, e.g. [Ac-D-Nal(2)^1, D-p-Cl-Phe^2,D-Pal(3)^3,Lys(Nic)^5,D-Lys(Nic)^6,Lys(iPr)^8, D-Ala^{10}]-, [Ac-D-Nal(2)^1,D-p-Cl-Phe^2,D-Pal(3)^3,Lys(Nic)^5, D-Lys(Nic)^6,Orn(iPr)^8,D-Ala^{10}]- and [Ac-D-Nal(2)^1,D-p-Cl-Phe^2, D-Pal(3)^3,Lys(Pic)^5,D-Lys(Pic)^6,Lys(iPr)^8,D-Ala^{10}]-GnRH, were very poor in releasing histamine from the rat mast cells (ED_{50} 90-300 μg/ml) but were extremely potent as antiovulatory agents (ED_{50} 0.51 μg/rat). In vivo side-effect profiles of these analogues have not yet been reported.

CONCLUSIONS

The chemical work on GnRH has led to potent agonist and antagonist analogues which have been shown to be effective in various fertility related disorders, contraception and hormone-dependent tumors. Some agonists have been formulated to give slow release of the peptide and some of these slow release formulations (including Zoladex depot) are already on the market.

The progress in the antagonist field has been slow. Considerable work still remains to be done in improving the side-effect profile and developing slow release formulations before these agents can be considered as useful drugs.

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3

ACTIVE REDUCED SIZE ANALOGUES

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INTRODUCTION

Although a large number of GnRH analogues have been synthesized, little has been reported on reduction of the size of these peptides. In addition, there are very few reports in the literature about active fragments of GnRH. J. Sandow and co-workers [1] tested fragments of buserelin using ovulation induction in the rat. They examined fragments wherein amino acids were removed sequentially from the N-terminus and they found that the (3-9)-buserelin was the sole fragment which still retained significant activity. It had 23% of GnRH activity. Although this activity represents only 0.2% of buserelin potency, we chose the (3-9)-peptide as a starting point for the design of reduced size GnRH analogues.

METHODS

Chemistry

All of the peptides were synthesized using solid phase methodology. All of the products were purified using HPLC and characterized using fast atom bombardment (FAB) mass spectroscopy, and amino acid analysis.

Biology

Receptor binding

A modified procedure from the literature [2] was utilized for the receptor binding assay. A rat pituitary plasma membrane fraction is prepared as a source of GnRH receptors, and ^{125}I-(Tyr^5)-leuprolide is made via chloramine-T oxidation and purified via ion exchange chromatography. Several concentrations of test compounds are coincubated for 120 minutes at 4°C with ^{125}I-(Tyr^5)-leuprolide and receptors until equilibrium binding, then bound tracer is separated from free via centrifugation. Data are reported as pK_I, the negative logarithm of the equilibrium

dissociation constant.

LH release

The ability of compounds to release luteinizing hormone (LH) from cultured pituitary cells [3, 4] was utilized for establishment of their activities. Pituitaries are dispersed with collagenase and hyaluronidase, and the cells are plated in culture plates for 72 hours. The cells are incubated with test compounds for 3 hours at 37°C, then the medium is harvested for determination of released LH via radioimmunoassay (RIA). The RIA utilizes ^{125}I-LH (ovine) as a tracer, immunoaffinity purified anti-rat-LH IgG from Dr. P.M. Conn, and rat LH (NIH-RP2) standard. Bound and free tracer are separated with immobilized protein A. The results for agonists are reported as a pD_2 which is defined as the negative logarithm of the concentration of agonist which results in 50% of the maximum release of LH produced by the compound. Antagonist results are reported as a pA_2, the negative logarithm of the concentration of an antagonist which shifts the LH release dose-response produced by leuprolide two-fold to the right.

RESULTS AND DISCUSSION

As an initial screen we selected rat pituitary receptor binding [2] (pK_1) and rat pituitary cell culture LH release [3, 4] (pD_2 for agonist, pA_2 for antagonist). In these assays, GnRH had a pK_1 of 8.90 and a pD_2 of 9.23 (Table 1).

Table 1. Rat pituitary receptor binding and LH release or inhibition by (3-9) GnRH analogues and indole-(4-9) GnRH analogues. Modifications of position 6.

X^3-Ser-Tyr-Z^6-Leu-Arg-ProNHEt

No.	X^3	Z^6	pK_1	pD_2	pA_2
1	Trp	DLeu	7.07		6.06
2	Trp	DTrp	8.33		7.24
3	(1)-Nal[a]	DLeu	7.30		
4	none	DLeu	5.36	NT[b]	NT
5	3-Indolepropionyl	DLeu	6.71	6.93	
6	3-Indolepropionyl	DTrp	8.34	7.35	
7	3-Indolepropionyl	D-(2)Nal[a]		8.77	
8	3-Indolepropionyl	DPhe	7.78		6.07
9	3-Indolepropionyl	DCha[a]	7.38		6.44
GnRH			8.90	9.27	
D-4-Cl-Phe^{1,2}DTrp^{3}DArg^{6}DAla^{10}			10.74		9.44

[a](1)-Nal = 1-naphthyl-3-alanine; D-(2)-Nal = D-2-naphthyl-3-alanine; D-Cha = D-3-cyclohexylalanine. [b]NT = not tested.

(3-9)-Leuprolide (1) had a pK_I of 7.07 and did not release LH, but antagonized its release with a pA_2 of 6.06. Similarly, D-Trp^6-(3-9)-leuprolide (2) was also a potent antagonist with pA_2 of 7.24. These results were surprising to us since (3-9)-buserelin was reported to be an agonist, and was active in the ovulation induction test in the rat [1]. For the (1)-Nal^3-(3-9)-leuprolide, which had a pK_I of 7.30, neither pD_2 or pA_2 could be determined suggesting that the peptide was probably degraded either chemically or by some proteolytic enzymes in the pituitary cell culture medium. (4-9)-Leuprolide (4) had a very weak binding with pK_I of 5.36 and was not tested further.

The first attempted structural modification was replacement of the Trp^3 of (1) with desamino-Trp or 3-indolepropionic acid to give compound 5 with virtually the same receptor binding affinity, indicating that the amino group is not essential for receptor binding. Removal of the α-amino group converts the molecule from antagonist to agonist. Affinity and potency increase as the residue at position 6 is changed from D-Leu to D-Trp and D-(2)-Nal; however, while the D-Leu (5) and D-Trp (6) forms are agonists, the D-(2)-Nal (7), as well as D-Phe (8) and D-Cha (9) are antagonists. The results indicate that not only the substituent at position 3 can influence the activity to be either agonist or antagonist, but also the substituent at position 6 has a similar effect. The highest affinity molecules of this series are about one fifth (for agonist 6) to equal (for antagonist 7) the affinity of GnRH. The affinity of antagonist 7 was one hundredth that of [D-4-Cl-$Phe^{1,2}$,D-Trp^3,D-Arg^6,D-Ala^{10}] LHRH, a potent, commercially available, decapeptide antagonist.

With affinity levels as high as these in a molecule that contains only six amino acids, we decided to explore the structure activity relationships a little further. The length of the aliphatic chain, connecting the indole group at position 4 to the molecule was varied (Table 2). Shortening the chain in (5) by one

Table 2. Rat pituitary receptor binding and LH release or inhibition by indole-(4-9) GnRH analogues. Variations of the indole chain length.

$(CH_2)_nCO$-Ser-Tyr-Z^6-Leu-Arg-ProNHEt

No.	n	Position 3	Z^6	pK_I	pD_2	pA_2
10	0	3-Indoloyl	DLeu	6.69		6.72
11	1	3-Indoleacetyl	DLeu	5.87		5.56
12	2	3-Indolepropionyl	DLeu	6.71	6.93	
13	3	3-Indolebutyryl	DLeu	6.91		6.72
14	3	3-Indolebutyryl	D-(2)Nal[a]	8.86		8.07

[a]D-(2)-Nal = D-2-naphthyl-3-alanine.

methylene (11) converted the compound from agonist to antagonist, however with a large loss in binding. Further shortening of the chain by an additional methylene (10) was still an antagonist, but receptor binding affinity was restored. Lengthening the chain in (5) by one methylene (12) also converted the compound from agonist to antagonist, but in this case maintaining the same binding affinity. [3-Indolebutyryl^3-D-(2)-Nal^6-Pro^9-NHEt]-(4-9)-LHRH (14) showed the highest binding affinity, which was equivalent to GnRH. Thus, once again we were able to shift between agonist and antagonist, this time solely by changing the number of methylene groups in the chain at position 3. The results suggest that there is an optimal size and shape for substituents at position 3 for potency and for agonist versus antagonist activity. These are unprecedented results, since previously antagonists have been reported by replacing residues 2, 3, and sometimes 1, and 10 with D-amino acids, or by deleting residue 2 in the decapeptide or nonapeptide GnRH analogue.

To further analyze the properties of the substituents at position 3, 1-naphthylpropionic acid, corresponding to the (1)-Nal^3 substitution, was placed at this position (Table 3).

Table 3. Rat pituitary receptor binding and LH release or inhibition by naphthyl (4-9) GnRH analogues. Modification of position 6 and variations of the naphthyl chain length.

X^3-Ser-Tyr-Z^6-Leu-Arg-ProNHEt

No.	X^3	Z^6	pK_1	pD_2	pA_2
14	1-Naphthylpropionyl	DLeu	7.60		6.60
15	1-Naphthylpropionyl	DTrp	9.28		8.74
16	1-Naphthylpropionyl	D-(2)Nal[a]	9.55		9.25
17	1-Naphthylpropionyl	DPhe	8.01		7.83
18	1-Naphthylpropionyl	DCha[a]	7.44		6.08
19	1-Naphthoyl	DLeu	6.12	6.00	
20	1-Naphthylacetyl	DLeu	7.17	6.85	
21	1-Naphthylacetyl	DTrp	8.65		8.18
22	1-Naphthylacetyl	D-(2)Nal	8.58		8.20
23	1-Naphthylacetyl	DPhe	8.01		7.83
24	1-Naphthylacetyl	DCha	7.44		6.08

[a]D-(2)-Nal = D-2-naphthyl-3-alanine; D-Cha = D-3-cyclohexyalanine.

Compounds with impressive receptor binding were obtained; the antagonist [N-(1-naphthylpropionyl),Ser^4,D-2- Nal^6,ProNHEt]-(4-9)-LHRH (16) had fifteen times higher affinity than GnRH and vitually the same antagonist potency (pA_2) as the potent standard antagonist, [D-4-Cl-Phe^{1,2},D-Trp^3,D-Arg^6,D-Ala^{10}]LHRH, even though it contained only six amino acids (versus 10 in the reference antagonist) and only one of D configuration (versus five in the reference antagonist). When the

length of the aliphatic chain connecting the naphthyl group to the molecule was shortened by one or two methylenes (20 and 19 respectively) once again the biological response switched, this time from antagonist to agonist. Consequently, we examined the influence of changes at position 6 on the biological activity of N-naphthylacetyl-Ser^{4}series. Again, as the size of the residue at position 6 was increased from D-Leu to D-Cha, D-Phe, D-Trp, or D-(2)-Nal, the molecule changed from an agonist to an antagonist with the expected enhancement in binding.

Table 4. Rat pituitary receptor binding and LH release or inhibition by naphthyl (4-9) GnRH analogues. Modification of position 3.

X^3-Ser-Tyr-Z^6-Leu-Arg-ProNHEt

No.	X^3	Z^6	pK_1	pD_2	pA_2
25	2-Naphthylacetyl	DTrp	8.43		7.80
26	1-Naphthylacrylyl	DLeu	7.30		6.69
27	1-Naphthoxyacetyl	DTrp	8.72		7.89
28	trans-O-Methoxycinnamoyl	DTrp	6.80		6.46
29	cis-O-Methoxycinnamoyl	DTrp	8.85		7.22
30	3-Pyridylpropionyl	DLeu	5.79	4.96	
31	3-Pyridylacrylyl	DLeu	5.82		5.72
32	1-Adamantylacetyl	DTrp	9.34		8.50

To further probe the properties of the side chain at position 3, we decided to examine several more radical modifications for Trp^3 in the (3-9)-GnRH series. Table 4 shows that a wide range of affinities were obtained, from very weak for the 3-pyridylpropionyl (30) to quite high affinity for the adamantylacetyl (32) with binding affinity three-fold higher than GnRH.

Finally, as a byproduct of the solid phase synthesis, several analogues were obtained with an O-benzyl group on the Ser^4 side chain (Table 5). The Ser^4(O-Bzl) peptides had only slightly different binding affinity from their respective parents, sometimes greater, sometimes lower, indicating that the free hydroxyl of the Ser^4 is not essential for binding. The biological activity results were more anomalous. In the case of 1-naphthylpropionyl^3-D-Leu^6 (33), the molecule was transformed from an antagonist to an agonist by the O-benzyl substitution. In another case, 1-naphthylacetyl^3-D-Leu^6, (35), the reverse effect happened; the O-benzyl group changed an agonist (20) into an antagonist (35).

measurement of LH by radioimmunoassay. Immediately thereafter ice cold methanol / 6 N HCl (1:200) solution was added (2 ml per well), and transferred to tubes with chloroform (1 ml and water (1.8 ml). Cells were scraped off using a rubber policeman. The extracts were sonified (for 5 min) and centrifuged (3000xg for 10 min). The upper water soluble phase containing IP was separated from the lower phase containing the lipid fraction (polyphosphoinositides). The radiolabeled inositol phosphates in the aqueous phase were determined by anion exchange chromatography on columns containing AG_1-x8 resins (BioRad, 200-400 mesh, formate form). Free inositol was eluted in the flow-through of distilled water and glycerophosphate by disodium tetraborate 5 mM/sodium formate 60 mM. Then sequential washes with 0.1 M formic acid containing 0.2, 0.4 and 1.0 M ammonium formate progressively eluted IP_1, IP_2 and IP_3. Five ml fractions were collected and measured after addition of 10ml scintillation fluid (Amersham) for myo-(2-^{3}H)-inositol.

Standard curves have been established using radiolabeled inositophosphates. Data are shown in counts per minute myo-(2-^{3}H)-inositol ± SD. Each data point consists of 3 independent control values and shows one out of two experiments. Statistical analysis was performed by Student's t test and, when for more than two means, analysis of variance was carried out.

Superfusion of cultured cells with phospholipases, arachidonic acid and leukotrienes

In contrast to static cultures, superfused cells are good for investigating substances with a short half-life or for determining the secretory profile of stimulants [21-24]. For that purpose, pituitary cells were cultured after enzymatic dispersion on cytodex beads and transferred to columns. They were allowed to recover for 90 min while being superfused with medium 199 Earle and 20 mM HEPES at a speed of 9.5 ml/min. Pituitary cells were stimulated by the addition of GnRH for 4 min dissolved in medium 199. As shown in Fig. 1, pulses of GnRH evoked a rapid release of LH with increasing amount of LH released per peak (area under the curve). GnRH was also added as a pulse at the end of other superfusions to demonstrate response of cells, and it served as a comparison for test substances. Arachidonic acid, and its lipoxygenase metabolites 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), (5s)-5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE), 15-hydroxy-5,8,10,14-eicosatetraenoic acid (15-HETE), leukotrienes A_4, B_4, C_4, D_4 and E_4 were dissolved in ethanol after deacylation and further diluted in incubation medium prior to use. These agents were added to media for 4 min with a final concentration of 0.01% ethanol. Phospholipase A_2, melittin, quinacrine and chloroquine were added in incubation medium. No effect of 0.01% ethanol on superfused rat pituitary cells was detected. Fractions were collected every 2 min and assayed for LH by radioimmunoassay kits RP-2 provided by the Hormone and Pituitary Program, Baltimore, USA. Data are presented

results obtained that a wide range of receptor binding affinities can be achieved with these hexapeptide GnRH analogues up to and exceeding the activity of GnRH itself. Even more important, by manipulating the group at position 3, one can change from agonist to antagonist almost at will. In examining the structure-activity relationships, it appears that a very precise size and shape of side chain substituent is required at position 3 to achieve agonist activity. If the group is too large, too small, too long, or too short, the molecule becomes an antagonist. The receptor binding site for position 3 seems to be formed in such a way that even though the response switches between agonist and antagonist quite readily depending upon changes in the size and shape of the substituent that occupies the site, these changes may have little effect upon the receptor binding affinity.

A novel result has been obtained upon modifying the nature of the side chain at position 6 in order to improve potency. While still performing its usual function of modulating receptor binding affinity in much the same way that it does in the nonapeptide or decapeptide GnRH analogues, i.e. D-(2)-Nal > D-Trp > D-Leu, in the hexapeptide series, position 6 somehow feeds back to the residue at position 3 to change the compound from agonist to antagonist as the side chain gets larger.

Finally, even modification of the serine side chain at position 4 by O-substitution with a benzyl group has been observed to trigger the switch from agonist to antagonist in either direction depending on the nature of the substituent at position 3. This suggests that it is not a direct effect of the benzyl group, but once again the feeding back of a conformational change, probably small, to the 3 position which influences the agonist to antagonist transition.

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4
INTRACELLULAR ASPECTS

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INTRODUCTION

The physiological actions and clinical applications of GnRH and its analogs have been extensively studied [1], but the mechanisms of GnRH and its regulation are not fully clarified yet. GnRH stimulates the biosynthesis and secretion of LH and FSH from the anterior pituitary gland. The intracellular pathway through which GnRH induces the release of pituitary gonadotropins is receptor-mediated and calcium-dependent. Cyclic nucleotides including cAMP [2,3] and later cGMP [4,5] were proposed to act as second messengers during GnRH-stimulated LH release. However, cAMP production does not correlate with GnRH-stimulated LH release and GnRH effects on LH release are not reproduced by elevation of intracellular cAMP concentrations [6]. Furthermore, while cGMP formation correlates well with the activation of LH secretion by GnRH and is also calcium-dependent, LH release has been shown to occur independently of cGMP production in GnRH-stimulated gonadotropes [7]. It is now believed that cyclic nucleotides play little if any part in the acute action of GnRH upon gonadotropin release [7,8], and that the effects of cAMP upon gonadotrope function are of a more long-term nature that may contribute to the facilitation of LH release during subsequent stimulations [9].

The stimulation of gonadotropin release by GnRH and high potassium concentrations is dependent upon the presence of extracellular calcium [10,11], raising the possibility of calcium influx being an important component of the activation process. Several recent studies have confirmed that increases in cytosolic calcium stimulate LH release [12-14], and that the actions of GnRH upon LH secretion and cGMP formation are related to the extracellular calcium concentrations [13]. The role of Ca^{2+} in GnRH action is well established. LH release is fully dependent on extracellular Ca^{2+} [14], but the binding of GnRH to its receptor is not a Ca^{2+}-dependent step [15]. The elevation of intracellular Ca^{2+} by different methods i.e. calcium ionophores, Ca^{2+}-loaded liposomes, veratridine provoked release of LH [16] and, in addition, the use of the fluorescent analog of the Ca^{2+}-specific

chelator EGTA allowed the demonstration of intracellular movement of Ca^{2+} in response to GnRH [17].

The observation that drugs which inhibit calmodulin also inhibit GnRH-stimulated gonadotropin secretion provides evidence that calmodulin may serve as an intracellular receptor for calcium following gonadotrope activation by GnRH [18]. In many tissues that are activated by extrinsic ligands changes of phospholipid metabolism have been observed which influence the availability and action of Ca^{2+}.

Various post receptor systems that may be linked to this mechanism (i.e. phospholipids and inositol phosphates, arachidonic and metabolites, protein kinase C) have been analyzed in regard to their function to mediate or regulate GnRH action on gonadotropin secretion.

METHODS FOR STUDYING POST-RECEPTOR SYSTEMS

Preparation of pituitary cells

Anterior pituitary glands of adult female Sprague-Dawley rats (200-250 g) were dispersed into a single cell suspension by trypsinization. Whole pituitaries from adult female rats were cut in small blocks and suspended in medium 199 containing 0.3% bovine serum albumin (BSA). The medium was replaced by 5 ml of 0.5% trypsin (type III, Sigma, Munich, FRG) in medium 199-0.3% BSA and incubated at 37°C for 15 min. Suubsequently the medium was decanted and incubations with 5 ml DNAse and soybean trypsin inhibitor (Sigma) were carried out for 1 min and 5 min, respectively. The tissue has been further incubated for 15 min each with 2 mM and 1 mM EDTA in Ca^{2+} and Mg^{2+}-free medium 199. Thereafter fragments were gently dispersed to a single cell suspension using a plastic Pasteur pipette. The cells were cultured for 2 days ($3x10^5$ cells/well) in medium 199 (Bio-Products, Berlin, FRG) containing 10% horse serum and 100 units penicillin and 100 µg streptomycin/ml. For certain experimental procedures pituitary cells were enriched for gonadotropes using centrifugal elutriation [19].

Measurement of inositol phosphate accumulation

GnRH-induced inositol phosphate (IP) production was measured to investigate the role of IP in the mechanism of gonadotropin secretion [20]. Two million cells per well were incubated for 4 days in Medium 199 Earle (modified, Seromed, Berlin/FRG) with penicillin 100 U/ml, streptomycin 100 mg/100 ml, 10% horse serum. On day 2 of incubation, $12x10^6$ cpm myo-(2^{-3}H)-inositol (10-20 Ci/mmol; Amersham) was added to each well. On day 4 medium was removed and replaced by stimulation medium (Medium 199 Earle, 20 mM HEPES, 0.01% BSA). Ten minutes prior to stimulation $LiCl_2$ was added, to prevent IP breakdown. Then GnRH was added at a final concentration of 10^{-7} M. The reaction was stopped by placing wells on dry ice. Medium was removed and saved for

Table 5. Rat pituitary receptor binding and LH release or inhibition by naphthylpropionyl-Ser^4(O-Bzl) and naphthylacetyl-Ser^4(O-Bzl) (4-9) GnRH analogues. The effect of O-substitution of Ser^4 with benzyl.

X^3-Ser(O-Bzl)-Tyr-Z^6-Leu-Arg-ProNHEt

No.	X^3	Z^6	pK_1	pD_2	pA_2
33	1-Naphthylpropionyl	DLeu	7.64	7.93	
34	1-Naphthylpropionyl	DTrp	8.20		7.10
35	1-Naphthylacetyl	DLeu	7.70		7.49
36	1-Naphthylacetyl	D-(2)Nal[a]	7.43		8.44
37	1-Naphthylacetyl	DPhe	8.46		7.98
38	1-Naphthylacetyl	DTrp	8.91		8.52

[a]D-(2)-Nal = D-2-naphthyl-3-alanine.

CONCLUSIONS

Several novel conclusions emerge from these studies that are very important for further development of LHRH analogues, especially reduced size and nonpeptidic versions. Figure 1 shows a schematic representation of the (3-9)-GnRH molecule. It is clear from the

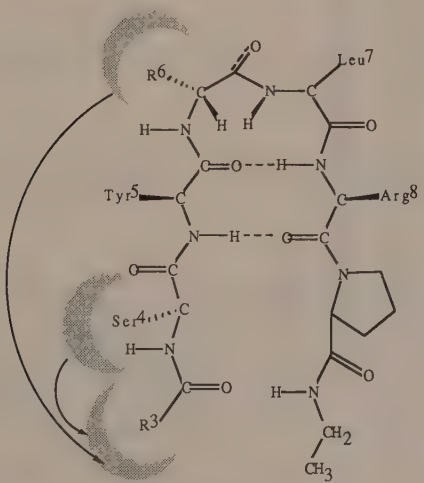

FIGURE 1 Schematic representation of the (3-9) GnRH hexapeptide series. The arrows represent the interactions that are believed to occur between position 6 and 3 that influence whether the molecule is an agonist or an antagonist

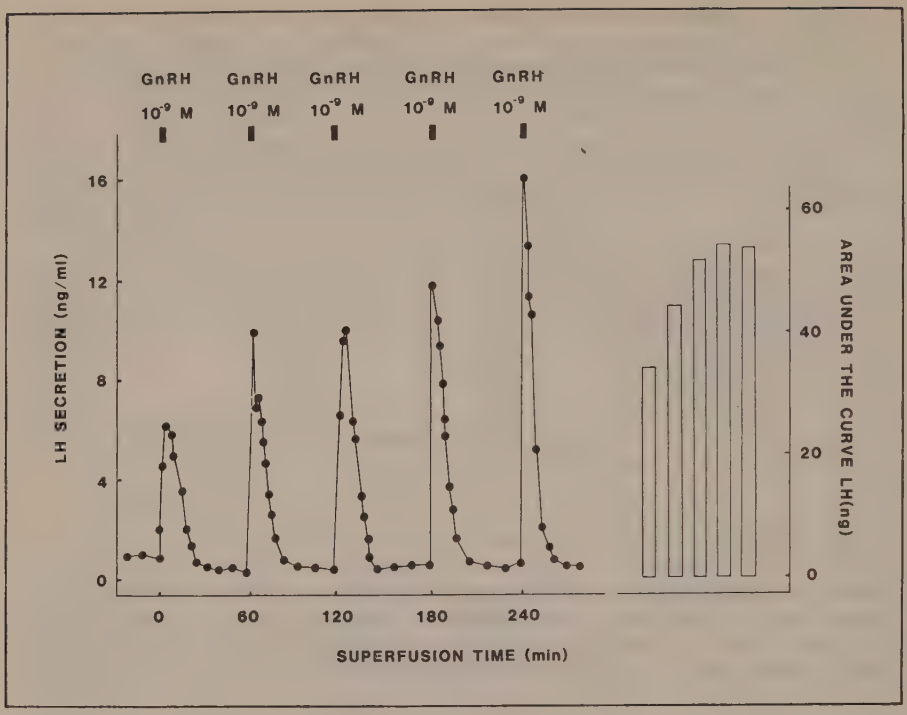

FIGURE 1 Stimulation of superfused pituitary cells by GnRH (10^{-9}M) pulses

as hormone release (ng RP-2/ml/2x10^7 cells per column) and show one representative experiment out of three sets.

Gonadotrope enrichment

Since the rat pituitary gland contains only about 10% gonadotropes, centrifugal elutriation (Fig. 2), a new method for enrichment of certain cell fractions [19], was used in some experiments. Thus, interference with other non-gonadotrope cell types of the pituitary could be diminished. Eight fractions were obtained by increasing the flow rate step-wise from 11.5 ml/min to 37.5 ml/min.

Franctions 6 and 7 contained 40-50% of gonadotrophs. Cells of each fraction were plated as monolayers and a GnRH challenge test was performed for 4 hours. Highest response was achieved by cells of fraction 6 and 7 (Fig. 3).

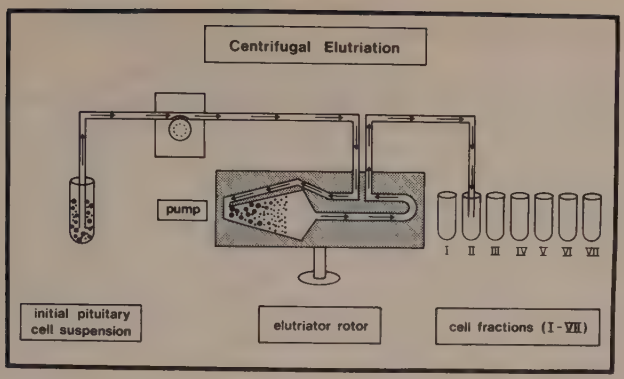

FIGURE 2 Centrifugal elutriation for preparation of gonadotrope-enriched cell populations

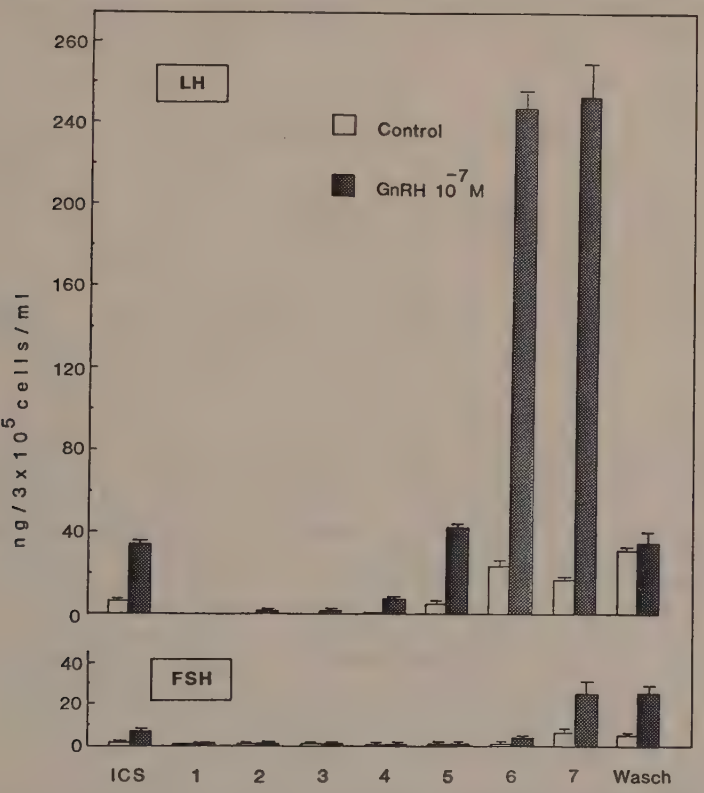

FIGURE 3 Cell fractions after centrifugal elutriation. GnRH (10^{-7}) challenge for 4 hours to stimulate LH and FSH secretion in initial cell suspension (ICS) and 8 fractions after elutriation. Cells were precultured for 48 hours

PHOSPHOLIPIDS, INOSITOL PHOSPHATES AND PROTEIN KINASE C

It is now recognized that activation of many tissues is accompanied by changes in the phospholipid metabolism which in turn influence the availability and actions of calcium within the target cell. One proposed sequence in ligand-activated cells attributes the earliest regulatory events to the phosphatidyl-inositol cycle, in which the breakdown of phosphatidylinositol (PI) to inositol phosphate and diacylglycerol is rapidly followed by the formation of phosphatidic acid (PA) and subsequently by re-synthesis of PI [26-28]. Recent findings have demonstrated the rapid change in phospholipid metabolism in rat pituitary cells stimulated by GnRH inducing the increase in ^{32}P labeling of PI [29,30]. Several of the intermediates formed during activation of the PI-cycle have the potential to act as intracellular messengers. The administration of the enzymes PL-C and A_2 which mediate the breakdown of polyphosphoinositides and the liberation of arachidonic acid, respectively, stimulates the release of LH (Figs. 4 and 5). The action of melittin (activator of PL-A_2) on

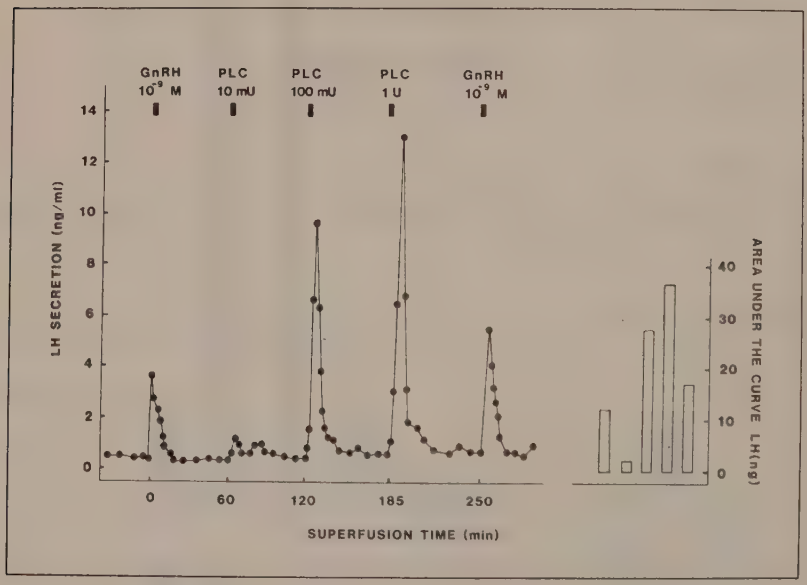

FIGURE 4 Stimulation of LH secretion in superfused pituitary cells by phospholipase C (PL-C)

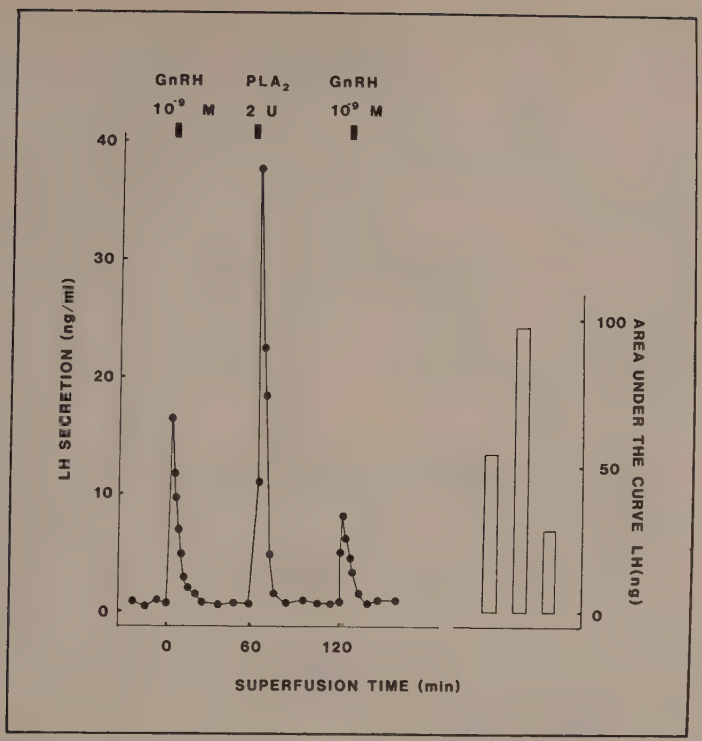

FIGURE 5 Stimulation of LH secretion in superfused pituitary cells by phospholipase A_2 (PL-A_2)

gonadotropin secretion is blocked by chloroquine (Fig. 6). PA can function as a calcium ionophore in several cell types [31], and could also be involved in the actions of GnRH on rat pituitary gonadotrophs [30]. GnRH stimulates ^{32}P incorporation into PI and PA release and cGMP production in a calcium-dependent manner in cultured gonadotrophs [30]. Apart from its calcium ionophore properties, PA is also a source of arachidonic acid, the major precursor of potential intracellular mediators formed via the cyclo-oxygenase and lipoxygenase pathways. However, the mechanistic role of arachidonic acid in secretory granule release remains to be clarified. In earlier studies, GnRH was found to cause arachidonic acid release from cultured anterior pituitary cells, and exogenous arachidonic acid was shown to stimulate LH release in vitro [8,32,33]. Recently, emphasis has been shifted to the breakdown of phosphoinositol-1,4,5-triphosphate (PIP_3), rather than of PI, as the major source of hormonal mediators such as inositol triphosphate and diacylglycerol [20,34].

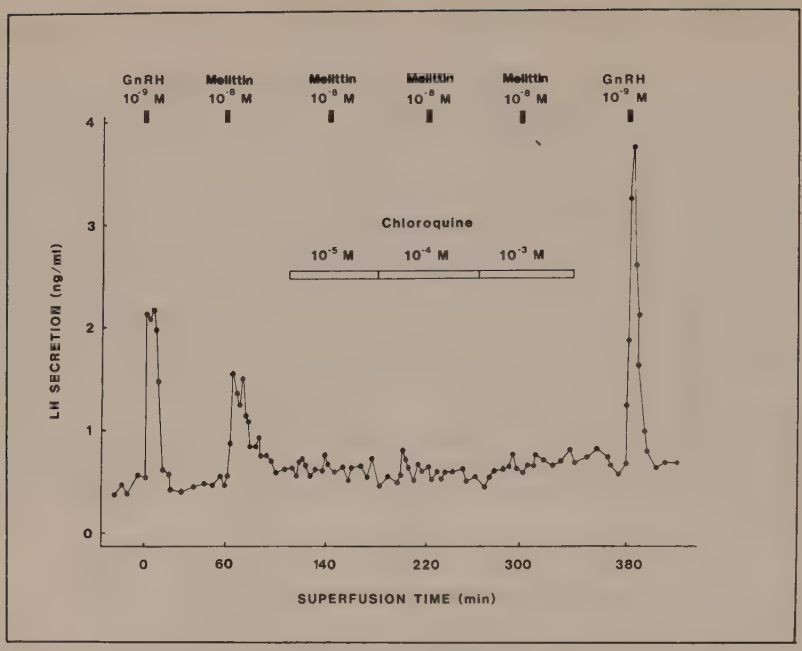

FIGURE 6 Stimulation of LH secretion in pituitary cell by melittin and the inhibition f its effect by the phospholipase A_2 blocker chloroquine

It is now evident that the diacylglycerol formed during phosphoinositide breakdown is a potent activator of the multifunctional protein kinase (protein kinase C) characterized by Nishizuka et al [35]. This calcium-and phospholipid-dependent enzyme, which is usually present in an inactive form, can be activated by the diacylglycerol that is transiently produced during ligand-stimulated phosphoinositide hydrolysis. In the presence of phosphatidylserine, the concentration of calcium required for activation of protein kinase C is markedly reduced by diacylglycerol, and the total activity of the enzyme is increased.

Protein kinase C is present in the pituitary, mostly as a soluble and partly as a particulate form [36-38]. Stimulation by GnRH induces translocation of protein kinase C from the cytosol to the membrane in the pituitary [36]. Similarly, the tumour promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA) activates protein kinase C but can elicit only a partial LH release when compared to GnRH.

In studies with myo (2-^{3}H) inositol prelabelled pituitary cultures we [20] and others [39-42] demonstrated that GnRH stimulation enhances the turnover of polyphosphoinositides. This stimulatory effect on inositol phosphate accumulation was shown to be time- and dose-dependent in enriched gonadotropes with maximal

5- to 8-fold increase achieved after 15 min [20]. Significant rise in IP_3 production was observed within 5 sec after GnRH administration (Table 1). Measurement of LH in these experiments

Table 1. Stimulation of inositol 1,4,5-trisphosphate (IP_3) production by GnRH in rat pituitary cells

Stimulation Time	IP_3 (cpm)	± SD
0 sec	2,429	± 147
5 sec	4,037	± 253
15 sec	3,922	± 308
30 sec	2,559	± 161
45 sec	3,220	± 173
1 min	2,840	± 224
3 min	3,313	± 508
5 min	3,150	± 144
15 min	3,304	± 138

revealed that it occurred later. The effect of GnRH on IP accumulation did not require extracellular calcium in contrast to gonadotropin secretion [20].

In further studies [43,44] the binding of inositol phosphates and the induction of Ca^{2+}-release from pituitary microsomal fractions were demonstrated. Bovine anterior-pituitary microsomal fractions exhibit high-affinity, saturable and reversible binding of inositol 1,4,5-(^{32}P)trisphosphate; 50% of the labelled ligand is displaced by 3.5 nM-inositol 1,4,5-trisphosphate, 0.5 nM-inositol 1,4-bisphosphate and 10 μM-ATP. Inositol 1,4,5-trisphosphate induces the release of Ca^{2+} from the microsomal vesicles (half-maximal effect at 290 nM), and its action is potentiated by inositol tetrakisphosphate (half maximal effect at 4 μM).

These findings on inositol phosphate production and action suggest that they may be a mediator of GnRH-induced gonadotropin release. There are, however, reports providing evidence that inositol phosphates are not the only mediators.

ARACHIDONIC ACID AND METABOLITES

Experiments using labelled arachidonic acid demonstrated the rapid production of metabolites in enriched gonadotrope fractions [46]. In addition, inhibitors of PL-A_2 provided evidence for the role of arachidonic acid and its active metabolites in GnRH-induced

gonadotropin secretion [47]. The stimulation of LH release by arachidonic acid has been demonstrated under both static and dynamic incubation conditions. Arachidonic acid causes an increase in LH secretion in static cultures of enriched gonadotropes [8], and has similar though attenuated effects in perifused gonadotropes (Fig. 7). In static cultures, the LH

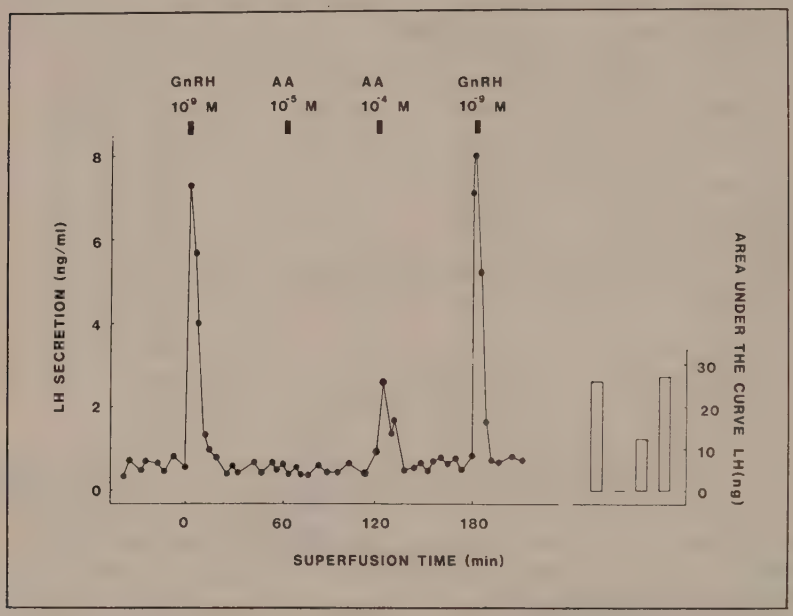

FIGURE 7 Stimulation of LH secretion in superfused pituitary cells by GnRH and arachidonic acid

response to GnRH is unimpaired following preincubation with arachidonic acid, indicating that the effects of the fatty acid are not due to non-specific toxic actions on hormone release. In the same context, addition of GnRH a few minutes after arachidonic acid in superfused gonadotrophs potentiated the LH response. The potency of arachidonic acid on gonadotropin release was about 4 orders of magnitude lower than that of GnRH, but the maximal stimulation of LH release was similar to that elicited by GnRH. Other fatty acids, including linoleic acid and oleic acid, had no effect on LH release, further indicating that the action of arachidonic acid does not reflect a non-specific effect on the gonadotrope [8].

In previous reports, GnRH action was found to include release of arachidonic acid [32,47], which serves as a precursor of several groups of intracellular mediators [32,47,48]. However, prostaglandins do not consistently stimulate LH release in vitro,

and GnRH-stimulated gonadotropin secretion is not prevented by cyclo-oxygenase inhibitors [49]. These findings suggest that cyclo-oxygenase products of arachidonic acid are not directly involved in the mechanism of GnRH action. Rather, the effect of prostaglandins on gonadotropin secretion appears to be indirect, via stimulation at the hypothalamic level [31,50].

Currently, two additional pathways of arachidonate metabolism: epoxygenase and lipoxygenase are being investigated. Epoxygenase metabolites of arachidonate, i.e. 5,6-epoxyicosatrienoic acid (5,6-EET) stimulate gonadotropin secretion in a range of 0.01-1 μM [51]. Lipoxygenase metabolites, especially leukotrienes, were found to be even more potent when added to piuitary cells in monolayer culture [32,52] and in a perifusion system [21-24]. The most effective doses were 10^{-10} and $3x10^{-11}$ M for LTA_4, LTB_4, LTC_4 and LTE_4. In Fig. 8, the

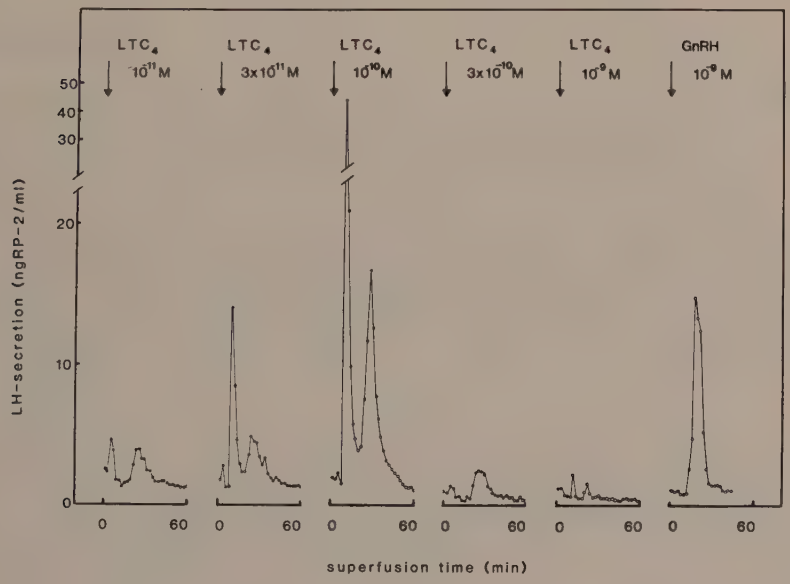

FIGURE 8 Biphasic stimulatory effect leukotriene C_4 (LTC_4) on LH secretion in superfused pituitary cells

effect of LTC_4 is shown. LTD_4 had no stimulatory action on gonadotropin release [23]. When higher concentration was continuously administered to superfused pituitary cells, gonadotropin secretion was suppressed 50% below basal values. Similarly, simultaneous pulses of LTA_4, LTB_4, LTC_4 and LTE_4 partially blocked GnRH-induced LH release (Fig. 9). These

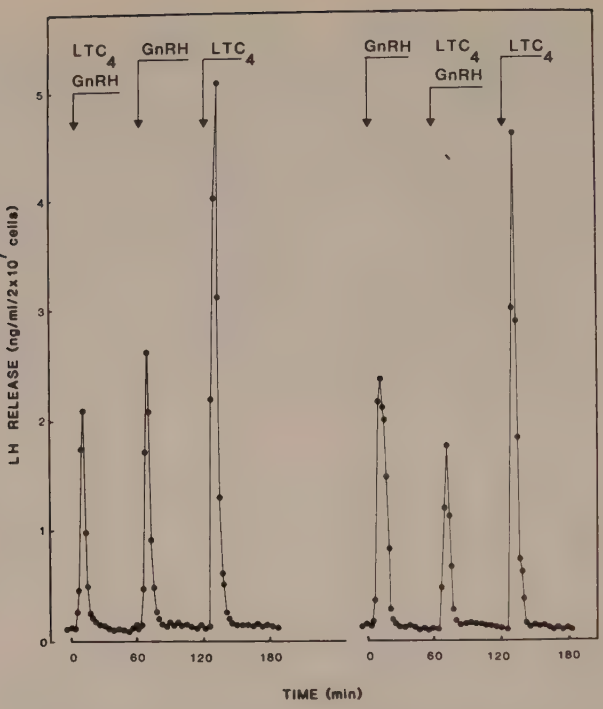

FIGURE 9 Stimulation of LH secretion in superfused pituitary cells by leukotriene C_4 (LTC_4, 10^{-13} M), GnRH (10^{-9} M) or both stimulants. The order of stimulation was changed in two different experiments. LTC_4 inhibited GnRH effect on gonadotropin secretion

findings suggest that leukotrienes can modulate ligand-stimulated hormone secretion in the pituitary [53].

The differential effect of leukotrienes may find its explanation in the following hypothesis. When leukotrienes are present in a certain narrow range of concentration (below 10^{-9} M) they can partially mimic the effect of GnRH and potently induce LH secretion. Higher concentrations, however, seem to be deleterious to basal hormone release.

When NDGA is administered prior to and during leukotrienes, endogenous synthesis is blocked, intracellular concentration of these mediators is very low and therefore exogenously added leukotrienes can exert an even more potent action on gonadotropes.

The understanding of the modulation of GnRH action is, however, more complex especially because not all post-receptor events are quite defined, yet. As observed by others [54] the activation of more than one second messenger pathway seems to be required to fully mimic the GnRH-induced gonadotropin secretion, demonstrated by the addition of arachidonate and phorbol esters.

Therefore the observation made in the present publication only indicates that leukotrienes disrupt the coupling of GnRH-receptor and post-receptor mechanisms.

Others have proposed a complex interpretation of dose-response curves for LTC_4 using a hormone/receptor/effector model [55].

Apart from leukotrienes a number of other potential second messenger mechanisms have been suggesed for GnRH-activation, i.e. Ca^{2+} [14], inositol phosphates [20,39-44], protein kinase C [36-38], epoxygenase metabolites of arachidonate [51]. It is up to the present yet unclear how post-receptor actions of GnRH are orchestrated, but previous data show that it is unlikely that inositol phosphates or protein kinase C alone are fully responsible for transduction of transmembrane signalling in gonadotropes [45,54]. Therefore we believe that the present observations regarding the dual mode of leukotriene action provide further evidence for the potential regulatory role of leukotrienes in pituitary hormone secretion.

CONCLUSIONS

The following mechanisms are postulated for the ligand-stimulated intracellular action of GnRH studied in this paper (Fig. 10). GnRH binds to its receptor at the membrane of gonadotropes and activates several intracellular second messengers. First, the hydrolysis of polyphosphoinositides (TPI, DPI) to PI provides inositol phosphates, especially IP_3, which serve as a signal for the mobilization of intracellular pool of Ca^{2+}. Second, a further product of polyphosphoinositide breakdown, diacylglycerol, activates protein kinase C causing translocation of this enzyme from the cytosol to the membrane. This multifunctional enzyme may also modulate phospholipid hydrolysis itself. Third, diacylglycerol could serve as a substrate for PLA_2, which releases arachidonic acid. In addition, phosphorylation by protein kinase C may induce mechanisms which activate PLA_2. Together with the above mentioned messengers certain specific pathways of the arachidonic acid metabolism could supply active products which could mediate gonadotropin release via granule fusion and exocytosis. GnRH-stimulated gonadotropin secretion is biphasic and there is now evidence that the initial phase of release may be mediated by the elevation of cytosolic free-Ca^{2+} and the production of potent metabolites of arachidonic acid, i.e. leukotrienes, whereas the activation of protein kinase C could be involved in the second phase of LH release. Recent studies have shown that post-receptor mechanisms of GnRH action can be modulated which are relevant for the understanding of clinically important phenomena such as mechanism of down-regulation for the treatment of sex-hormone dependent diseases as well as the action of steroids during menstrual cycle or in hormonal contraception. The role of protein kinase C in the mechanism of gonadotrope down-regulation is controversial [45], but some data suggest that the activation of this enzyme may be involved in pituitary desensitization [56].

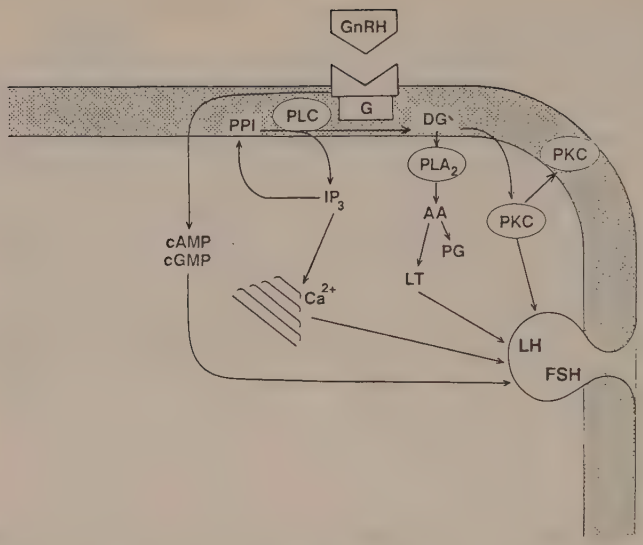

FIGURE 10 Summary of post-receptor actions of GnRH in pituitary cells.

G, GTP-binding protein; DG, diacylglycerol; PIP_2, phosphatidylinositol 4, 5-bisphosphate; IP_3, inositoltriphosphate; PG, prostaglandins; LT, leukotrienes; PK-C, protein kinase C; AA, arachidonic acid

In addition, leukotrienes could be responsible for an intracellular feedback mechanism as shown by the inhibitory effect of leukotrienes on GnRH-induced LH-release, whereas leukotrienes alone are very potent stimulators of gonadotropin secretion [53]. It has been suggested that leukotrienes may modulate GnRH-induced inositol phosphate accumulation.

Steroids are known modulators of gonadotrope function [57]. A detailed analysis of the inhibitory action of progestins has been performed [58] and some experimental data suggest that progestins may also interfere with post-receptor mechanisms, such as IP production [58].

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5
EXTRAPITUITARY ACTIONS

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INTRODUCTION

Extrapituitary actions of LHRH and its analogues in vivo have been suggested by the presence of LHRH-like peptides and LHRH receptors in various extrapituitary tissues and by observed biological effects in vitro or in hypophysectomized animals after administration of LHRH analogues.

Hypothalamic hormones are not only detected in the hypothalamus, but also in the central nervous system and in different normal and tissue tumors. LHRH-like peptides have been found in tissues derived from the pancreas, in the gonads, in cultures of Sertoli cells, in the placenta and in human biological fluids (Table 1); in addition they have been found in different human tumors including insulinomas, choriocarcinomas and breast cancers. The meaning of the presence and local production of hypothalamic peptides in peripheral tissues is not clear, but probably they have a local regulatory function by autocrine or paracrine mechanisms of action.

Table 1. LHRH-like material has been demonstrated in:

- Hypothalamic neurons
- Brain
- Spinal ganglia
- Pineal gland
- Pancreatic islet cells, insulinoma
- Ovary, testis
- Placenta, Choriocarcinoma
- Mammary carcinoma cells
- Biological fluids, - plasma
  - urine
  - milk
  - ovarian follicular fluid
  - semen

Specific binding sites for LHRH have been characterized in the anterior pituitary, brain, testis, ovary, placenta, adrenals and in various tumors (Table 2). In general, human extrapituitary tissues contain LHRH receptors which have a considerably lower affinity for LHRH than do the human pituitary or the rat testis and ovary [1]. It has been suggested that LHRH receptors in various tumors are induced by carcinogenic transformation of normal cells in view of the absence of these receptors in most non-neoplastic tissues [2].

Table 2. LHRH (analogue) binding sites

High affinity (Kd 10^{-8} -10^{-9} M)	Low affinity (Kd 10^{-5} -10^{-8} M)
- Rat pituitary	- Human corpus luteum
- Human pituitary	- Human ovarian tumors
- Rat testis (Leydig cells)	- Placenta
- Rat ovary: interstitial cells	- Adrenals
luteal cells	- Mammary carcinoma cell lines
granulosa cells	Human primary breast cancers
	- Dunning R-3327 H prostate tumor
	Prostate tumor cell lines
	- Leiomyoma
- Discrete brain regions	

Direct biological effects of LHRH have been demonstrated in vitro or in hypophysectomized animals on cells from the gonads, accessory sex organs, placenta, kidney and on mammary and prostate tumor cells (Table 3). However, species variation exists concerning the direct action of LHRH and its analogues [3]. Initially, there was great interest in the extrapituitary actions on normal tissues (see reviews [1,3-6]); later, more interest was shown in the direct growth inhibitory effects on tumor cells.

TESTIS

Hsueh and Erickson [7] showed for the first time that treatment with LHRH analogues decreased the testicular weight and testicular steroidogenesis in hypophysectomized male rats. After demonstration of direct inhibition of androgen secretion by LHRH in vitro, from cultured rat testicular cells, Hsueh et al showed that the inhibitory effect of LHRH on testicular androgen production occurs at sites distal to the formation of cAMP and pregnenolone. They and others attributed the effect as being due to decreases in the activity of the enzymes 17-alpha-hydroxylase and 17,20-desmolase[8,9]. There was a dose-dependent inhibition

of gonadotropin-stimulated Leydig cell production of testosterone by LHRH and its agonists [8-10]. LHRH receptors have been identified by several authors in Leydig but not in Sertoli cells [10-12]. With respect to ontogeny, LHRH receptors were not detectable in rat fetal testes but were present post-natally and increased markedly with age [10]. Administration of LHRH agonists decreased testicular gonadotropin receptors [3,7], but administration of LH reduced both basal and LHRH-stimulated LHRH binding sites in cultured Leydig cells [10].

Table 3. Direct effects of LHRH analogues

1. Interstitial, luteal and granulosa cells of rat ovaries in vitro.
2. Rat testicular cells in vitro.
3. Placental tissue in vitro.
4. Mammary tumor cells in vitro.
5. Prostate tumor cells in vitro.
6. Inhibition of estrogen-induced growth of the uterus in ovariectomized hypophysectimized rats in vivo.
7. Inhibition of androgen induced growth of accessory sex organs in hypophysectomized castrated rats in vivo
8. Inhibition of androgen stimulated enzyme activity in mouse kidney in vivo.

The concentration of LHRH in peripheral blood is too low to postulate direct effects of hypothalamic LHRH on gonads. Therefore several laboratories have attempted to isolate gonadal LHRH-like peptides which may serve as the ligand for specific gonadal LHRH receptors. LHRH or LHRH-like molecules can indeed be isolated from rat testes (see review [5]). Such LHRH-like factors have been detected in extracts of rat gonads and in spent media from cultured Sertoli cells [13]. The observations of high molecular weight LHRH-like factors [14] together with the reported testicular concentration of LHRH of 1 pg/testis [15] and the existence of a precursor molecule for LHRH, alleged to be present in gonads [16], represent the available experimental evidence for intratesticular production of LHRH or LHRH-like factors [5]. Therefore a local paracrine regulatory function of gonadal LHRH or LHRH-like molecules was proposed [13]. However, in human testes LHRH-like material and receptors have not been demonstrated [17]. In addition, several groups do not find evidence for direct inhibitory effects of LHRH agonists on testicular steroidogenesis in men. Administration of LHRH agonists did not inhibit hCG-induced testosterone secretion in patients with gonadotropin deficiency [18] or hCG-induced intratesticular enzyme activities in patients with disseminated prostatic cancer [19]. It needs to be pointed out, however, that it is still conceivable that large doses of hCG/hLH may have overcome a small direct gonadal effect of the administered LHRH analogues [20]. The lack of detection of LHRH receptors in human testis could also be due to

down-regulation of LHRH receptors in the human gonads by the high circulating levels of bioactive LH [10]. Nevertheless, most evidence points to a predominant pituitary site of action of LHRH analogues in the human male.

OVARY

As for the rat testis [7], LHRH analogues inhibit FSH-induced changes in ovarian function in hypophysectomized rats [21]. In addition, LHRH and its agonists have been reported to have direct inhibitory effects on rat ovarian steroidogenesis in vitro [21,22]. Direct stimulatory and inhibitory effects of LHRH analogues on granulosa, luteal and theca cells have been demonstrated [3]. The early effects are mostly stimulatory and the later effects inhibitory [1,3]. In granulosa cells the major action of LHRH appears to be stimulation of 20 alpha-hydroxy-steroid dehydrogenase which causes increased metabolism of progesterone. LHRH also increases aromatase activity in these cells [3]. On the other hand, LHRH agonist delayed hCG-stimulated accumulation of cyclic AMP in porcine granulosa cells obtained from medium follicles [23]. This delay of cyclic AMP accumulation has been suggested to be responsible for the inhibition of progesterone production by ovarian cells [23]. In addition, treatment with high concentrations of LHRH can also induce ovulation and oocyte maturation in hypophysectomized rats, which is associated with the ability of LHH to stimulate plasminogen activator activity in cultured granulosa cells [3]. With respect to mechanism of action, recent studies have shown that LHRH action in the ovary is dependent upon calcium mobilization and probably operates through stimulation of phospholipid turnover and activation of protein kinase C [24].

In the rat ovary, specific high affinity LHRH receptors have been identified in granulosa and theca cells [3,22]. These binding sites mediate the inhibitory effects of LHRH and its agonists on gonadotropin-stimulated estrogen, progestin and androgen biosynthesis. In addition, LHRH appeared to increase its own receptor in follicular or luteal tissue which is functionally active [25]. LHRH also stimulates prolactin receptor content in rat ovaries [24].

With respect to the effects of LHRH and its analogues on the human ovary the data are conflicting. Tureck et al [26] reported that an LHRH agonist is able to inhibit the secretion of progesterone by cultured human granulosa cells in a dose dependent manner. The effect was prevented by the addition of an LHRH antagonist. On the other hand Casper et al found no effect of LHRH or its agonist on progesterone production by granulosa cells [27]. Comparable conflicting data have been reported with respect to the presence of LHRH receptors in human ovaries. Low affinity LHRH receptors have been identified in human corpus luteum [28]. Bramley et al [29] showed marked variation of specific binding of LHRH agonist to human corpora lutea at different stages of the

luteal phase. In contrast, Clayton and Huhtaniemi [17] reported the absence of LHRH receptors in corpus luteum.

Substances with LH releasing properties in extracts from rat ovaries were first described by De Jong et al [30]. Later on, the rat ovary appeared to contain significant amounts of LHRH-like protein [31]. In contrast, Fraser et al[1] did not find an LHRH-like substance in ovaries. Recently Aten et al [31] detected equivalent amounts of LHRH-like activity in both human and rat ovaries, which was not native LHRH. The LHRH-like material in the human ovary appeared similar to that of the rat ovary. The meaning of this ovarian LHRH-like material remains speculative, but indicates that direct effects of LHRH analogues on human ovaries is possible. However, most evidence suggests that the primary antifertility effect of LHRH analogues in primates is through an action on the pituitary [1].

ACCESSORY SEX ORGANS

Uterus

LHRH agonists have been reported to have an inhibitory action upon the estrogen-stimulated growth of the uterus in ovariectomized hypophysectomized rats [32,33]. LHRH analogue treatment does not alter the estradiol receptor content either in vivo or in vitro [34]. Reddy et al [34] showed that LHRH analogues inhibited estradiol-induced ornithine decarboxylase and glucosamine-6-phosphate synthase activities in the uterus of rat. Although there is no evidence for direct effects of LHRH agonists on the human uterus, specific binding sites for LHRH in uterine leiomyoma have recently been demonstrated [35].

Prostate and seminal vesicles

LHRH agonists have been shown to have direct anti-androgenic effects by inhibition of androgen-induced growth of seminal vesicles and prostate [33]. In addition, similarly to the uterus, LHRH inhibited testosterone-stimulated ornithine decarboxylase activity in the ventral prostate of rat [34]. LHRH receptors have been demonstrated in an experimental prostate tumor [36], but thus far not in the normal human prostate or in primary human prostate tumors.

ADRENALS AND KIDNEY

Adrenals

Specific LHRH receptors have been detected in mouse [37] and rat adrenals [25]. Recently Eidne et al [38] demonstrated a 60 K molecular weight LHRH receptor in solubilized rat adrenal membranes by a ligand-immunoblotting technique.

Kidney

LHRH analogues have antisteroidal activity in the rat kidney. Androgen responsive mouse kidney beta-glucuronidase induction could be inhibited by LHRH analogues [39]. Recently, Prasad et al [40] showed that an LHRH agonist caused significant inhibition of androgen-induced renal ornithine decarboxylase and poly(A)-polymerase activity.

PLACENTA

Khodr and Siler-Khodr [41] have reported the presence of LHRH in human placenta and have also shown stimulatory effects of LHRH on the synthesis and secretion of human chorionic gonadotropin. Addition of LHRH antibodies [5] or LHRH antagonist [42] to the medium completely blocked the action of LHRH on placental hCG and progesterone secretion. Tan and Rousseau [43] demonstrated LHRH and high molecular weight LHRH-like compounds in human trophoblasts.

Specific binding sites for LHRH have been demonstrated in placental tissue [1,44-46]. It has been postulated by Iwashita et al [46] that placental low affinity binding sites may be activated by high local concentrations of a placental LHRH-like peptide. A gene for LHRH has been isolated from the human placenta [47]. It seems that the placenta is the only other tissue besides the pituitary where LHRH has probably a regulatory role in the human female. The potential clinical relevance of these findings might be the application of LHRH antagonists as post-coital contraceptives and abortifacients in humans [6].

TUMOR CELLS

Breast cancer

The presence of direct effects of LHRH analogues on mammary tumor cells is supported by the following findings: 1) presence of LHRH in milk; 2) LHRH-like immunoreactivity in mammary tumor cells; 3) specific LHRH binding sites in human mammary cell lines and human primary breast tumors; 4) direct biological effects in vitro.

Several authors found LHRH in milk [48-50]. Sarda and Nair [49] demonstrated that the LHRH concentration in milk was 6-fold that determined in plasma. They concluded that passive diffusion of LHRH may only contribute to a very small extent towards this milk LHRH. However, the values quoted in the literature for milk LHRH are relatively low.

In 1980 Seppälä et al found LHRH-like material in breast ductal carcinoma [51]. Recently Bützow et al [52] demonstrated immunoreactive LHRH in 4 mammary carcinoma cell lines. In 2 cell lines hCG was also demonstrated, but could not be stimulated by exogenous LHRH [42,45].

Specific LHRH binding sites of low affinity (Kd 10^{-5}-10^{-6}M) have been identified in 7 mammary cancer cell lines i.e. MCF-7 [53-55], T-47-D [54], MDA-MB-231 [54,55], MDA-MB-157 [52,55], ZR-75-1 [55], SK-Br-3 [55], and UCT-Br-1 [55]. In human primary tumors, Eidne et al [56] found specific LHRH receptors in 67%. The incidence was higher in ductal (86%) than in lobular carcinoma (22%). They found no relationship with the estradiol receptor (ER), progesterone receptor (PR) or age. Binding sites for LHRH were not detected in normal breast tissue. Kaufmann et al [57] found LHRH binding in 38 out of 44 (86%) tumors. Patients with values of $\geq$ 3.0 fmol/mg protein (48%) were classified as LHRH receptor positive. In contrast to Eidne, these authors found higher values in ER-positive tumors than in ER-negative tumors. Miller et al also reported low affinity binding sites (Kd 10^{-5}M) in some primary tumors [1].

Several direct biological effects on mammary tumor cells in vitro have been demonstrated. In 1982 Corbin [58] and Matzuzawa and Yamamoto [59] showed direct growth inhibitory effects of LHRH agonists on mouse mammary tumor cells in vitro. In 1983 we demonstrated inhibition of estradiol stimulated growth of human breast cancer cells in vitro by the LHRH agonist buserelin [60-62]. One year later Wiznitzer and Benz [63] showed growth inhibitory effects by a LHRH agonist with respect to prolactin-stimulated growth of another cell line. In 1985 and 1986 Miller et al [53,54] observed not only inhibition of growth, but even regression in number of tumor cells by doses from 10^{-9} to 10^{-6} M buserelin. In 1986 we reported that tamoxifen partly can abolish the inhibition of estradiol stimulated growth of MCF-7 cells by buserelin [64,65]. Buserelin also inhibited the expression of prostaglandin receptors induced by estradiol [64,65]. There were no effects on the levels of cytoplasmic or nuclear ER, no change in the patterns of secreted proteins nor on the secretion of polypeptides with EGF-like activities. Both Miller et al [53,54] and our group [61,64,65] showed that concomitant administration of LHRH antagonists prevented the growth inhibitory effects of LHRH agonists on MCF-7 cells, and that the presence of estradiol concentrations higher than 30 pmol/l ($\geq$ 10^{-10}M) or insulin in the medium abolished the growth inhibiting effects of LHRH agonists. Native LHRH also appeared capable of inhibiting tumor cell growth but required higher concentrations. Furthermore, Miller et al [54] using different steroid receptor positive and negative cell lines showed parallelism between sensitivity to LHRH agonist and both responsiveness to estrogen and levels of ER. In contrast, Eidne et al [55] found no effect of LHRH agonists on various cell lines, but showed LHRH antagonists (10^{-5} to 10^{-6} M) to inhibit [^{3}H]thymidine-uptake within 3 hours leading to inhibition of cell growth after 6 days. Also de Launoit et al [66] showed a rapid antimitogenic effect of LHRH analogue treatment. An LHRH agonist caused a decrease of the labeling index from 12-48 hours after administration in ovariectomized hypophysectomized mice with MXT mammary tumors. On the other hand, we found no effect of LHRH agonist administration on percentage of MCF-7 cells in S-phase of

the cell cycle, when the cells were cultured in serum-free medium in the absence or presence of estradiol for up to 48 hours.

Negative results of LHRH treatment of tumor cells have also been reported. Furr and Nicholson [67] found no anti-estrogenic effects of LHRH agonists in ovariectomized immature rats. Nicholson et al [68] were not able to detect tumor growth inhibiting effects of intratumoral LHRH agonist implants in DMBA rat mammary tumors. They also did not find inhibition of estradiol supported growth of MCF-7 xenografts in ovariectomized nude mice using the LHRH agonist goserelin. Recently Wilding et al [69] reported no effect of high dose LHRH agonists (10^{-6}M) on doubling time of estradiol-stimulated MCF-7 cells. However, their negative results can be explained by the use of a high dose of estradiol (10^{-8}M), which may have overruled the suppressing effects of the LHRH agonists.

PROSTATE CANCER

In 1986 we reported inhibition of androgen (10^{-11}M) stimulated growth of LNCaP human prostate cancer cells in vitro by 0.8 μM buserelin [65]. Schally and Redding [2] also demonstrated inhibition of this cell line by 10^{-2} to 10^{-6} M tryporelin. In addition these authors reported inhibition of 2 other prostate tumor cell lines (DU-145, PC-3) and inhibition of the growth of the Dunning R-3327H-G8-A1 clonal prostate adenocarcinoma cell line at doses as low as 10^{-8} of the agonist D-Trp^6-LHRH. Specific receptors for LHRH were demonstrated in the Dunning rat prostatic tumor [36]. Recently Scaletsky et al [70] were able to demonstrate LHRH receptors with low affinity (Kd of 10^{-6}M) in both LNCaP and DU145 cell lines. Interestingly, in the LNCaP line, incubation with an LHRH agonist led to a dose dependent increase in receptor binding. However, thus far in human primary prostate tumors neither LHRH-like molecules nor LHRH receptors have been found (Reubi et al, personal communication).

OVARIAN CANCER

Kullander et al [71] reported inhibition by LHRH agonist of the growth of rat ovarian tumors autografted under the splenic capsule. Recently Emons [72] demonstrated specific low affinity binding sites in 80% of human primary ovarian tumors. In some small series of patients with progressive disease after first-line chemotherapy, treatment with LHRH agonist showed growth inhibiting effects in several patients [71,73,74]. However, it is unclear whether this tumor growth inhibition was caused by direct or indirect endocrine effects. Direct inhibition on testosterone secretion from arrhenoblastoma cells cultured in vitro in the presence of an LHRH agonist has been shown [75].

CONCLUSIONS

LHRH-like peptides and specific binding sites for LHRH are demonstrable in various normal and malignant extrapituitary tissues. However, these specific binding sites in extrapituitary tissues are of low affinity with the exception of those detected in rat testis and ovary. The importance of the presence of LHRH-like molecules in different extrapituitary tissues remains unclear. Autocrine or paracrine actions have been suggested.

There exists a large species variation with respect to direct actions of LHRH and its analogues.

Although evidence exists from experimental animals for a direct antigonadal effect of LHRH agonists, such data in humans remain controversial. The demonstrated direct effects of LHRH analogues in vitro are probably of low significance in vivo, especially in humans.

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6

FIRST GENERATION GnRH ANTAGONIST: OVULATION INHIBITION BY WEEKLY TREATMENT

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INTRODUCTION

Although inhibition of ovulation in the form of combination oral contraceptives has been the most effective strategy for achieving reversible pharmacological fertility control in women, there is increasing interest in contraception achieved through GnRH analogs that act on the pituitary to induce a hypogonadotropic status [1]. GnRH agonists do so after a transient stimulatory phase that lasts from 1-3 weeks [2,3]. In contrast, GnRH antagonists lack stimulatory activity, causing prompt reduction of gonadotropin secretion [4].

To explore the use of a GnRH antagonist to achieve inhibition of ovulation in a practical and potentially clinically useful manner, nonhuman primates were tested in a protocol of intermittent GnRH antagonist administration during the menstrual cycle. Our initial findings prompted a second study aimed at clarifying the mechanism by which the preovulatory LH surge was blocked.

MATERIALS AND METHODS

Experimental subjects

Fifteen adult cynomolgus monkeys with regular ovulatory menstrual cycles were subjected to GnRH antagonist treatment. The methods of feeding, housing, blood collection and drug administration were described previously [5].

Experimental design

Study I. The GnRH antagonist [N-AcD-pCl-Phe^{1},D-pCl-Phe^{2}, D-Trp^{3},D-Arg^{6},D-Ala^{10}]GnRH was a generous gift from the Contraceptive Development Branch, NICHHD. Based on previous dose studies demonstrating profound suppression of serum LH and FSH levels by this agent at a daily dose of 1 mg/kg, [4] a higher dose was chosen for weekly administration. Since the dominant follicle

was likely to be selected by cycle day 7 [6], the intermittent dose of GnRH antagonist might achieve its ablation, thereby causing initiation of a new follicular phase. The GnRH antagonist was suspended in sesame oil and injected im.

Eight monkeys were used to test ovulation inhibition. Drug administration began on day 3 after spontaneous onset of menses; a single dose of 10 mg/kg was injected. This initial injection was followed at weekly intervals with single doses of 5 mg/kg on days 10, 17, and 24. During the first 42 days after initiation of the pretreatment menstrual flow, femoral blood was obtained on alternate days. On day 43, the frequency of blood drawing was decreased to twice weekly through day 64, when the study was terminated. Throughout sera were harvested and frozen for subsequent RIA. During the 4 weeks of GnRH antagonist treatment, laparoscopies were performed at weekly intervals to assess ovarian status.

Study II. To clarify the mechanism by which the GnRH antagonist blocked estrogen-induced LH surges in study I, normal ovulatory monkeys received the GnRH antagonist daily (2 mg/kg in sesame oil) from either days 2 through 9 or days 2 through 6 of the menstrual cycle, respectively, and estradiol (E_2) benzoate (50 µg, iv: n=4) was given on day 6. Because the weekly treatment regimen used in study I gave suppression of pituitary function followed by transient recovery in a series of four episodes, we selected a daily regimen in study II to strive for steadier, more consistent conditions for estrogen challenge and GnRH test of pituitary function. Accordingly, these estrogen or GnRH challenge tests provided an evaluation of pituitary gonadotropin secretory status during the GnRH antagonist regimen; serum LH concentrations served as the principal end point. The same test was given to three control monkeys on day 6 of the normal menstrual cycle.

RIAs for E_2, progesterone (P_4), LH, and FSH were performed as described previously [6,7]. The monkey LH RIA had an intraassay variation of 1.9-5.0% and an interassay variation of 9.3-18.7%.

Tests for significant differences ($P<0.01$) of the intervals from the onset of menstruation to the next LH surge and/or ovulation and the intermenstrual interval were performed using the F statistic [8].

RESULTS

During the 4-week course of intermittent GnRH antagonist treatment in study I, there were no ovulation sites seen at laparoscopy. Moreover, the absence of ovulation was confirmed by the lack of serum P_4 elevations; also, no LH surges were found.

Mean peripheral serum E_2 levels immediately before and on the day after the four GnRH antagonist treatments are shown in Fig. 1. Interestingly, there was no sustained suppression of E_2 in serum.

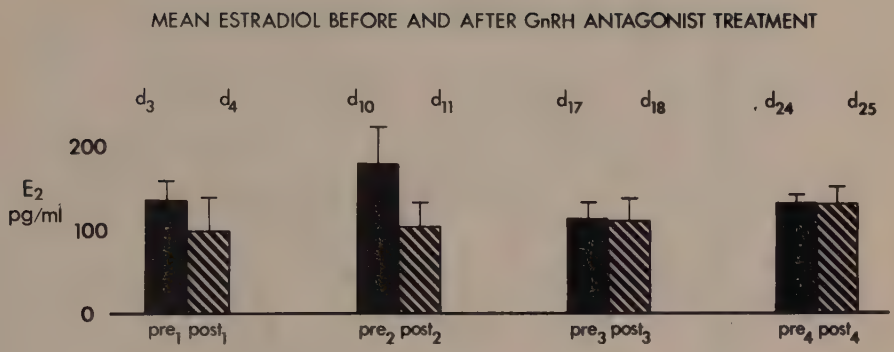

FIGURE 1 Mean ± SEM peripheral E_2 levels immediately before (▨) and the day after (▨) the four weekly treatments with GnRH antagonist in intact adult monkeys. There was no significant suppression of E_2 levels during these intermittent treatment intervals. d_3. Day 2, etc.

Results from a representative animal with ovulation inhibition and absence of LH surges during treatment as well as resumption of an ovulatory ovarian cycle after treatment are shown in Fig. 2. After discontinuation of treatment with the GnRH antagonist, serum E_2 levels rose within 3 weeks and were followed by timely LH surges in six of eight monkeys.

Ovulation was indicated by typical P_4 elevations in serum and an ensuing luteal phase of normal length. The levels of E_2 and P_4 in the circulation were consistent with single ovulations. The mean interval from the last GnRH treatment to the subsequent preovulatory E_2 peaks and LH surges was approximately 14 days among the 6 monkeys who resumed ovulatory menstrual cycles during the study interval. The remaining 2 monkeys did not resume regular menstrual cycles until nearly a month after completion of this study. Since blood collections did not extend beyond day 64 of the study, we do not know whether these resumed menstrual cycles were ovulatory.

Figure 3 illustrates pituitary refractoriness (three of three monkeys) to the E_2 benzoate challenge test given on cycle day 6 during the daily GnRH antagonist regimen spanning days 2 and 9 (study II). Notice the initial negative feedback effects of exogenous estrogen on LH and FSH secretion, the absence of familiar preovulatory-like LH surges, and finally, the resumption of apparent ovulation about 3 weeks later.

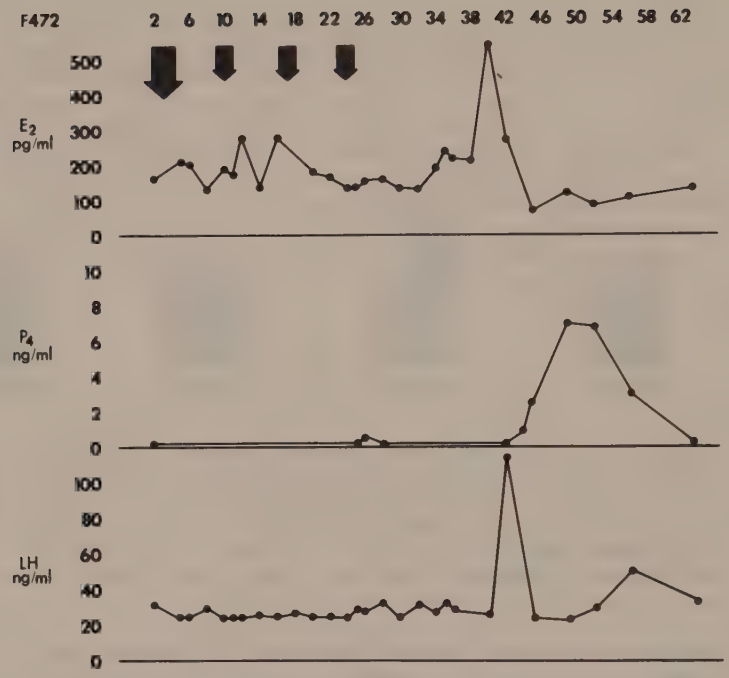

FIGURE 2 Peripheral serum E_2, P_4, and LH levels in a monkey (six of eight) during and after weekly administration of GnRH antagonist (arrows). Note that within about 15 days after the last GnRH antagonist treatment, high serum E_2 levels and a LH surge occurred, followed by an increase in serum P_4. Day 1 was the spontaneous onset of menstruation (top line). Compared to the normal interval from menstruation to ovulation (21) the delay of apparent ovulation and next menses associated with GnRH antagonist treatment was highly significant ($P<0.01$).

In contrast, administered GnRH (as a 50 μg bolus dose given on cycle day 6) during the daily GnRH antagonist regimen elicited unambiguous LH responses (four of four monkeys) of seemingly normal strength and duration (Fig. 4). All responses exceeded 4-fold elevations and did not differ significantly from the response of control monkeys given the same iv bolus dose of GnRH (Fig. 5).

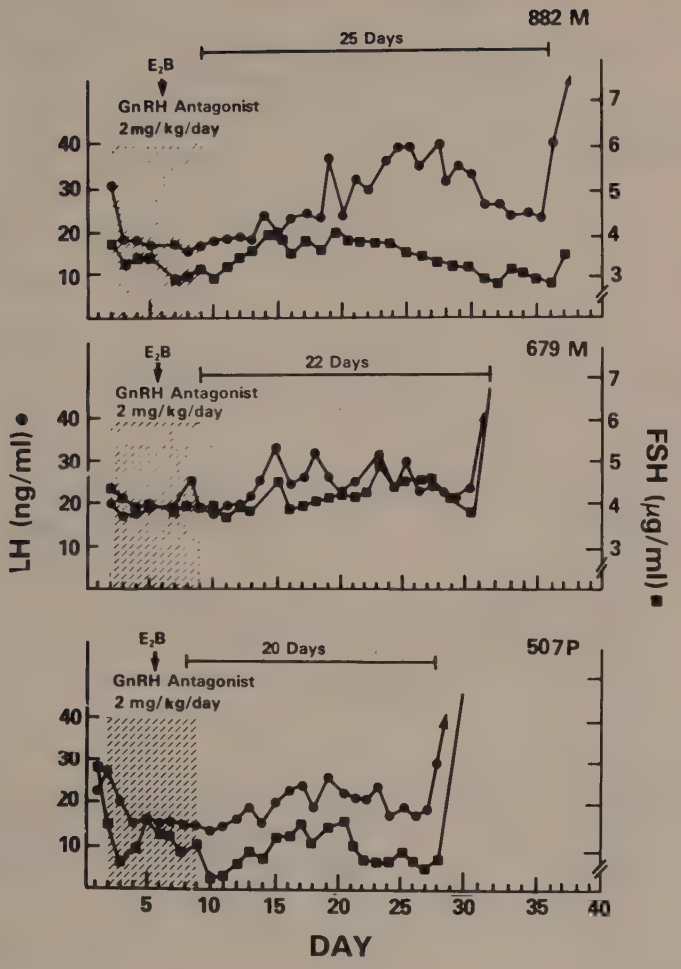

FIGURE 3 E_2 benzoate (E_2B) challenge tests in monkeys receiving the GnRH antagonist (2 mg/kg day, im) from menstrual cycle days 2-9. Note the absence of gonadotropin surges, indicative of pituitary refractoriness to estrogen-positive feedback. Without the inhibitory effects of the GnRH antagonist, such exogenous estrogen treatment leads to preovulatory-like LH surges within 48 h [21-23].

In six of the eight monkeys, ovulation resumed promptly after the last GnRH antagonist administration; the first post-treatment estradiol peak occurred 14.3 ± 3.8 (± SEM) days after the

final dose. The interval for resumption of ovulatory menstrual cycles after the final GnRH injection is summarized in Table 1.

Table 1. Post-treatment interval to first LH surge and resumption of menstruation

No. of Monkeys	Days	
	E_2 Peak	Menses
6 of 8	14.3 ± 3.8[a]	30.5 ± 3.4
2 of 8	Unknown	>64; <88

[a]Mean ± SEM

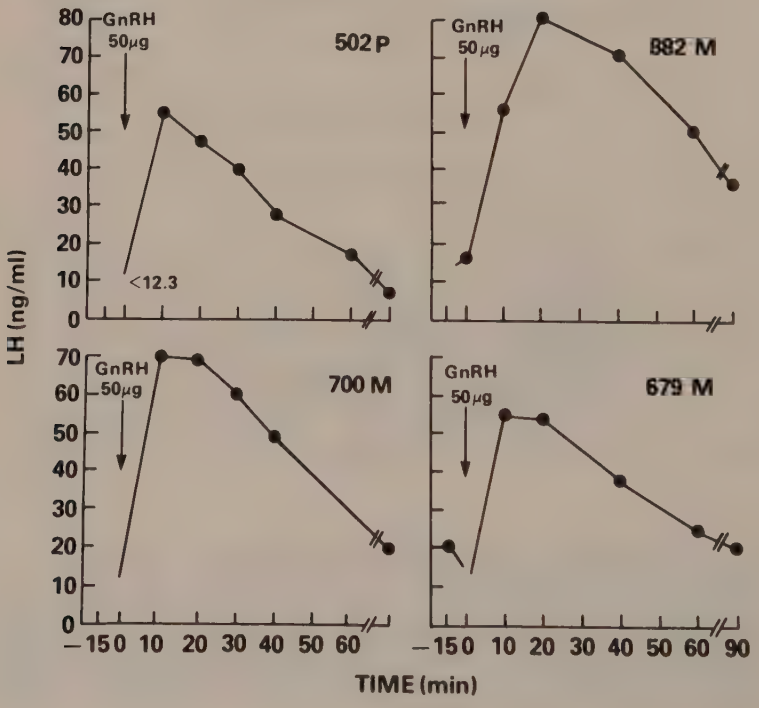

FIGURE 4 Serum LH levels in four monkeys receiving a GnRH bolus dose (50 μg, iv) during concurrent administration of GnRH antagonist.

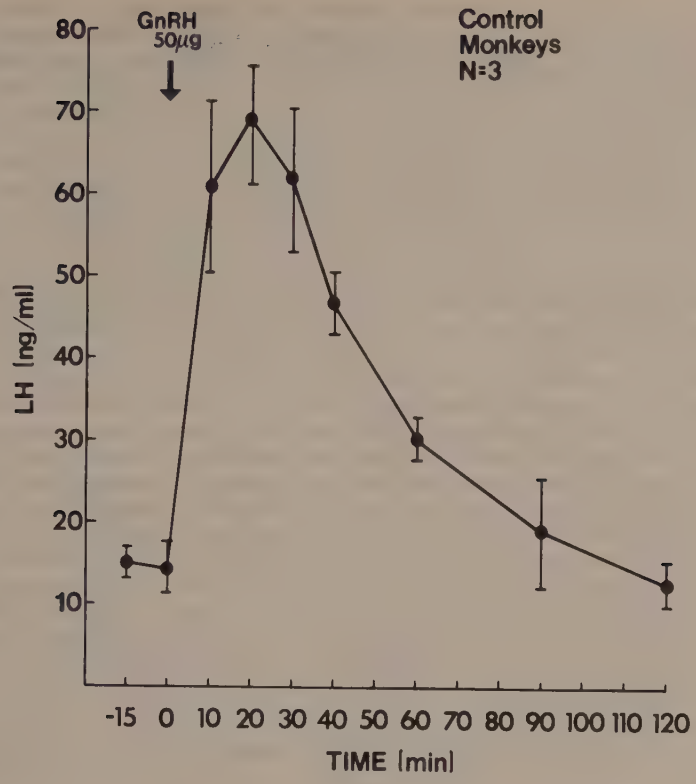

FIGURE 5 Mean ± SE serum LH responses to a GnRH bolus dose (50 μg, iv) in three control monkeys on day 6 of the normal menstrual cycle.

DISCUSSION

GnRH agonists effectively inhibit ovulation in women and monkeys when given in either daily or intermittent regimens [2, 3, 9-11]. Characteristically, GnRH agonists initially stimulate pituitary gonadotropin secretion and, in turn, enhance ovarian steroid production [11]. This action is transient; typically, both hypogonadotropic and hypogonadal conditions are achieved within 14-21 days [3,10,12,19]. In contrast, potent GnRH antagonists immediately reduce gonadotropin secretion [4].

Monkeys previously having regular ovarian/menstrual cycles had no ovulation (32 and 32 treatment intervals) when the GnRH antagonist was administered once weekly. After cessation of GnRH antagonist therapy, ovulation resumed promptly in 6 of 8 monkeys. That some monkeys regained ovulatory status as soon as 8-10 days

after the final dose of GnRH antagonist suggests that a weekly treatment interval may be near the upper limit for maintenance of ovulation inhibition at the dose tested here. These observations correlate well with earlier experience in monkeys [13, 14] and women [15] undergoing surgical ablation of the putative dominant follicle, in whom destruction of the largest visible follicle delayed ovulation by causing reinitiation of a new follicular phase. Perhaps each weekly dose of GnRH antagonist caused atresia of the newly selected dominant follicle. The intermittent elevations of E_2 in serum may reflect its secretion by newly recruited follicles in the next growing cohort [13].

It is not clear why two monkeys given the GnRH antagonist had longer delays before resuming regular menstrual cycles. However, their suppressed status ended less than 60 days after the cessation of treatment.

In regard to the mechanism by which the intermittent GnRH antagonist regimen prevented ovulation, the data from study II indicate that the estrogen-positive feedback for the LH surge was negated. During the normal follicular phase, E_2 benzoate will induce midcycle-like LH surges within 48 h; the GnRH antagonist clearly negated this estrogen-positive feedback [16-18]. In contrast, the response to GnRH was normal. It is possible that the iv bolus dose of GnRH displaced the GnRH antagonist from gonadotrope receptors, so that the exogenous GnRH elicited normal LH secretory responses. An alternative interpretation is that the dose of GnRH antagonist was submaximal, since previously we achieved a medical hypophysectomy-like status with only 50% of the dose used here [4].

In considering potential clinical applications derived from these studies, the avoidance of a severely hypoestrogenic milieu in these monkeys is encouraging in view of the concern that sustained reductions in E_2 levels might have undesirable sequelae, including hot flashes, urogenital atrophy, and osteoporosis in young women who might desire fertility control by a GnRH antagonist [19,20]. Even so, if clinical studies are undertaken, the effects of long-term unopposed estrogenic stimulation and the potential risk of endometrial carcinoma prompt consideration of periodic progestin supplementation during intermittent GnRH antagonist administration to accommodate sloughing of proliferated endometrium.

In summary, these studies demonstrate the potential feasibility of a weekly GnRH antagonist regimen to reliably inhibit ovulation in the primate ovarian/menstrual cycle. Further studies are warranted to assess the feasibility of intermittent GnRH antagonist therapy for reversible fertility control in women.

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7

SECOND GENERATION GnRH ANTAGONIST: CHARACTERIZATION OF PITUITARY RESPONSE IN MONKEYS

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INTRODUCTION

Previous studies have forecast potential therapeutic uses of GnRH analogs in reproductive endocrinology [1,2,3]. Until now, most human and animal data have been obtained using agonists of GnRH [4-13]. However, the development of useful antagonists of GnRH is now approaching clinical investigation. In some circumstances, these antagonists offer more therapeutic promise than the agonists.

GnRH antagonists are analogs of GnRH having a high affinity for the pituitary GnRH receptor, but with no intrinsic biological activity to stimulate gonadotropin secretion [14]. Persistent administration of a GnRH antagonist suppresses basal levels of gonadotropins creating a reversible medical hypophysectomy [15]. Not only do GnRH antagonists almost immediately suppress basal gonadotropin secretion, they also blunt the response of the pituitary to an estrogen challenge test [16-19].

We designed the following experiment in order to evaluate: (1) dose-response characteristics of a newer generation GnRH antagonist; (2) correlation between initial basal (tonic) gonadotropin levels and days needed to achieve pituitary suppression; and (3) pituitary response to a GnRH challenge test before, during, and after GnRH antagonist administration.

MATERIALS AND METHODS

Four ovariectomized (8 weeks or more) female rhesus monkeys (Macaca mulatta) were assigned to this study. Animal husbandry, blood collection techniques, and assays have been previously described [20]. Reagents for FSH/LH assays were obtained from the Hormone Distribution Office, NIAMDD, Bethesda, MD. Coefficients of variation within and between assays were 7.1% and 11.8%, respectively.

DRUG ADMINISTRATION

The GnRH antagonist used in this study was [N-Ac-D-Nal(2)^1,D-pCl-Phe^2,D-Pal(3)^3,Arg^5,D-Glu(AA)^6,D-Ala^{10}] [21]. The antagonist was dissolved in sesame oil and administered intramuscularly. During the first three days, 2 ml of vehicle was given (Fig. 1). From days 4 to 6, monkeys received 0.3 mg/kg/day of the GnRH antagonist; the dose was increased to 1.0 mg/kg/day from days 7 to 9, and to 3.0 mg/kg/day from days 10 to 12. Lastly, vehicle only was given again from days 13 to 15 during post-treatment recovery.

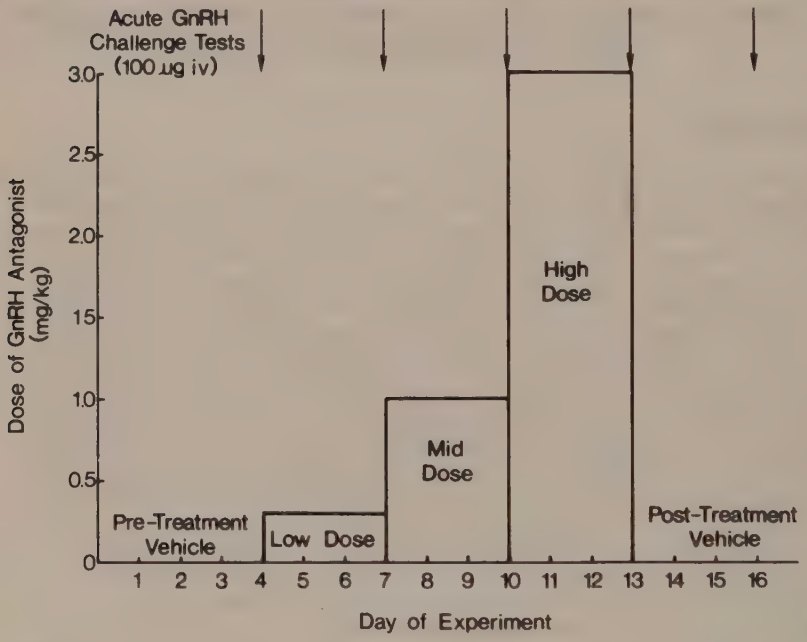

FIGURE 1 Experimental protocol. Four ovariectomized female monkeys received GnRH antagonist i.m. over 9 days in increasing doses of 0.3, 1.0 and 3.0 mg/kg/day. GnRH (100 µg/iv) was administered on experiment days 4, 7, 10, 13 and 16; blood samples were drawn at 0 and +30 minutes

GnRH CHALLENGE TEST

GnRH (100 µg, intravenously) was given as a bolus injection via a catheter inserted into the saphenous vein. The GnRH challenge test was performed on days 4 (control), 7, 10, 13 (treatment) and 16 (recovery). On these occasions, GnRH was injected 60 minutes before administration of the GnRH antagonist.

SERUM COLLECTION

Blood was drawn daily from the femoral vein on day 1 until experiment day 16 before the injection of GnRH antagonist, as well as at 0 and +30 minutes relative to GnRH administration. Therefore, the levels of LH and FSH reported for a certain day reflect the effect of the antagonist given on the previous day, as well as the acute effects of GnRH challenge tests given intermittently.

LH and FSH levels are expressed in ng/ml. Statistical differences were measured through analysis of variance and correlation coefficients for changes in tonic gonadotropin levels in serum and the response to GnRH challenge tests [22].

RESULTS

Tonic Gonadotropin Profiles

Initial levels of plasma gonadotropins were in the ovariectomized range. After 3 days vehicle administration, there were no meaningful changes in the tonic levels of gonadotropins. However, within 24 hours of administration of 0.3 mg/kg of the GnRH antagonist, there was a decrease in serum levels of FSH and LH. After 3 days of GnRH antagonist treatment at 0.3 mg/kg, tonic FSH and LH concentrations in serum decreased to near minimal detectable limits (Fig. 2). After 3 days of treatment with 1 mg/kg/day of the GnRH antagonist, LH and FSH levels were suppressed below assay detection limits in all monkeys. The decrease was highly significant for both FSH and LH ($p<0.005$). As expected, when the dose of antagonist was increased to 3 mg/kg/day, gonadotropins remained suppressed. During the recovery interval, despite administration of vehicle alone, tonic serum gonadotropin levels in all monkeys remained undetectable.

Table 1. Duration of antagonist treatment before FSH/LH levels became undetectable

Monkey#	Initial FSH (ng/ml)	Days Until FSH/LH Below Detection Limits	Initial LH (ng/ml)
		FSH/LH	
S172	46	2 / 2	120
0698	88	3 / 3	140
U531	420	6 / 4	192
713H	440	6 / 5	260

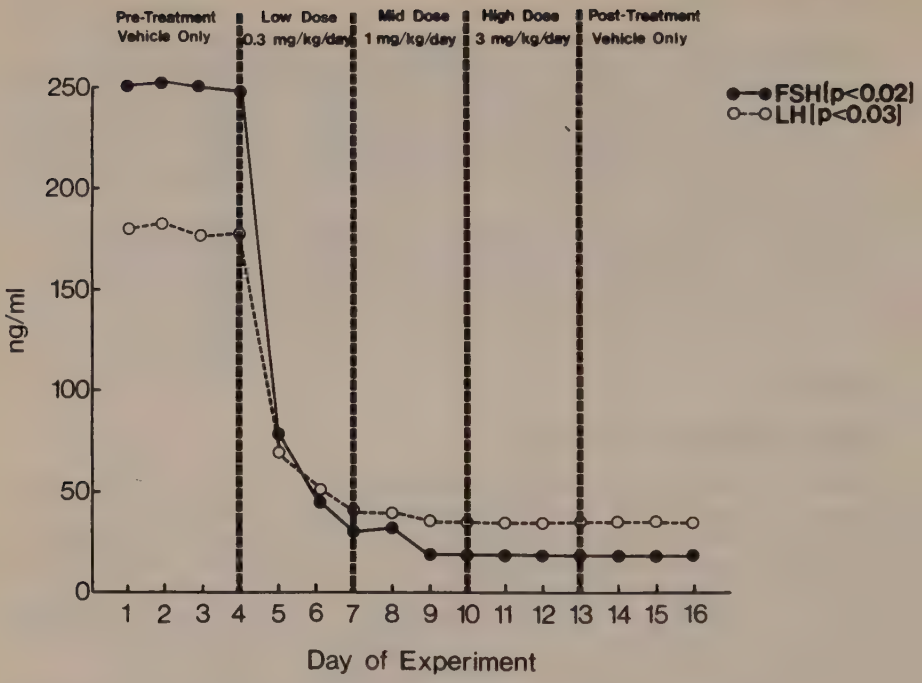

FIGURE 2 Tonic serum concentrations of FSH and LH before, during, and after GnRH antagonist treatment. Each point represents the mean of 4 animals. The decrease in serum FSH and LH throughout the study was significant ($p<0.05$)

The time needed to achieve suppression was not the same in all monkeys. Whereas some needed only two days of treatment to achieve full suppression, others required as much as 6 days of treatment with the GnRH antagonist in order to reach a 'medical hypophysectomy" state. Table 1 demonstrates a correlation between the initial tonic levels of gonadotropins and the days of antagonist administration necessary to achieve suppression. That is monkeys whose pituitaries quickly submitted to GnRH antagonist suppression had begun with lower tonic levels of FSH and LH in serum compared to females having higher initial levels of pituitary gonadotropins. Accordingly, there was a high correlation ($r=0.992$) between initial FSH levels and the interval of treatment necessary to achieve maximal suppression ($p<0.01$).

RESPONSE TO GnRH CHALLENGE TEST

After 3 days of treatment with 0.3 mg/kg of GnRH antagonist, bolus administration of 100 µg of GnRH intravenously elicited the release of gonadotropins not dissimilar from responses under pretreatment conditions (Fig. 3). Upon increasing the dose of

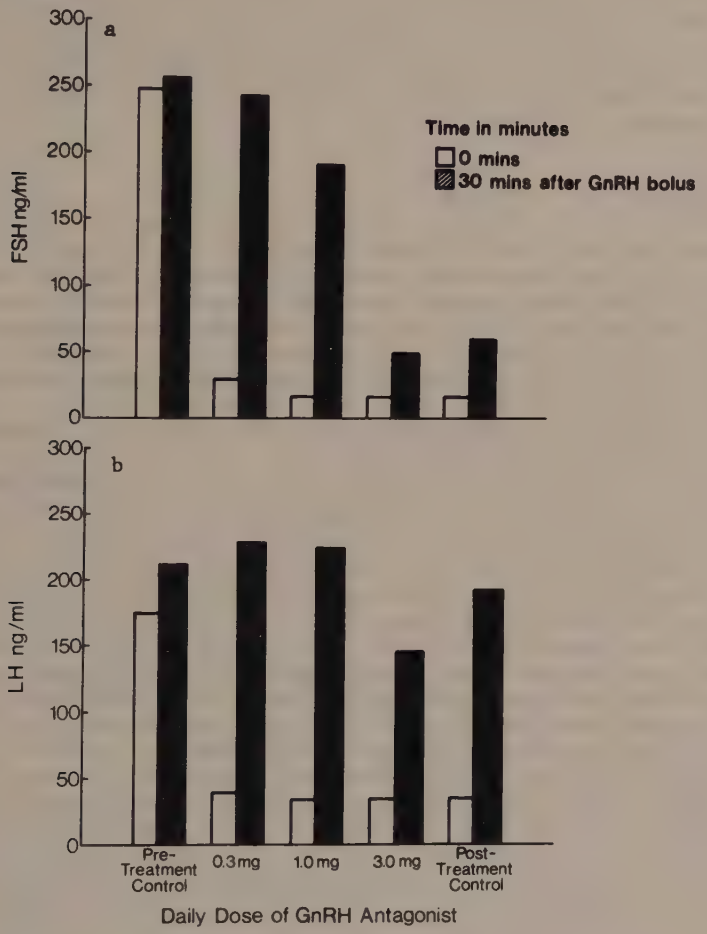

FIGURE 3 Serum concentrations of FSH (3a) and LH (3b) before and 30 minutes after a GnRH challenge test (100 µg/iv). Each bar represents the mean gonadotropin level ± SEM, n=4

GnRH antagonist to 1 mg/kg, although tonic secretion of LH and FSH was markedly suppessed, acute responses to the GnRH challenge were again similar to pretreatment tests. Despite 9 days of treatment with increasing doses of the GnRH antagonist and with

gonadotropins consistently below detection limits in all monkeys, the GnRH challenge still elicited a small release of FSH ($p<0.01$) (Fig. 3a); and the LH secretory response was only slightly inhibited ($p<0.05$). Interestingly, as rapidly as 3 days post-treatment, although tonic gonadotropins remained suppessed, there was partial restoration of FSH secretory response to the GnRH challenge test, and acute LH release was already fully recovered.

Importantly, we found no histamine release effects either locally or systemically during 9 days of GnRH antagonist therapy at doses up to 3.0 mg/kg/day. This dose is up to 10-fold more than that required to achieve a persistent state of medical hypophysectomy.

DISCUSSION

Our data indicate that within 24 hours of a single dose of 0.3 mg/kg of GnRH antagonist, profound suppression of FSH and LH concentrations in peripheral serum was evident. Depending on the initial tonic levels of LH and FSH, gonadotropins were suppressed below detectable limits after 2 to 6 days of GnRH antagonist administration. Tonic FSH/LH levels remained suppressed during the study interval, even 3 days into the post-treatment recovery interval. These data demonstrate that this potent antagonist of GnRH has a long duration of action.

GnRH antagonists have significant therapeutic advantages over agonists in that they almost immediately inhibit gonadotropin secretion without inducing an initial rise in gonadotropins, as is characteristic of GnRH agonist during the first two to three weeks of treatment [23]. There have been relatively few clinical trials using GnRH antagonists because of the lack of a potent GnRH antagonist without side effects. One of the main side effects of GnRH antagonists was reported by Schmidt et al [24], who demonstrated that administration of certain antagonists of GnRH to rats produced transient edema of the face and extremities, including a cutaneous anaphylactoid-like reaction [25]. This side effect was due to release of histamine by the drug. Among the most potent analogs associated with this histamine release phenomenon are those having structures that include a D-arginine at position 6. The antagonist used in the present study has an arginine substitution in position 5 with a D-glutamine in position 6. Although our observations were not specifically directed at this issue, the combination of structural features avoided discernible histamine release in monkeys.

We found a strong correlation between tonic levels of gonadotropins and the days of GnRH antagonist treatment necessary to achieve full suppression. Using a different GnRH antagonist, Cetel et al [26] reported that the net decrement in gonadotropin concentrations was correlated with tonic values of LH and FSH in women. Thus, a higher dynamic state of pituitary gonadotropin secretion portends greater relative resistance to suppression by the GnRH antagonist in women and monkeys. Therefore, when

evaluating the potency of a GnRH antagonist, it may be important to take into consideration the endocrine milieu of the individual.

Although we were able to decrease the levels of gonadotropins within 24 hours after administration of a single dose (0.3 mg/kg) of GnRH antagonist and were able to achieve undetectable levels of LH and FSH after 6 days of treatment in all monkeys, even 9 days of GnRH antagonist treatment did not fully block FSH or LH secretion during a GnRH challenge test. Indeed, LH secretory responses were hardly affected. This agrees with a recent study done by Kenigsberg and Hodgen [17], but is in contrast with a study done by Balmaceda et al [19] in which bolus injections of GnRH 18 hours after administration of another GnRH antagonist elicited a blunted release of gonadotropins. A similar response was seen in adult male monkeys by other authors [18]. Importantly, we performed the GnRH challenge test 23 hours after GnRH antagonist administration. Thus, the pituitary may have had enough time to recover from the acute effects of the antagonist.

Because some recovery of pituitary FSH/LH secretion in response to the acute GnRH challenge test was seen post-treatment, we postulated that low doses of GnRH antagonist may suppress tonic gonadotropin secretion, while the synthesis and storage of LH and FSH are replenished and sustained concurrently. This would allow for maintenance of the ready releasable pool of gonadotropins, as when doses of GnRH are given. Administration of high doses of GnRH antagonist may not only suppress the secretion of basal gonadotropins, but also block the synthesis and storage of gonadotropins in the anterior pituitary.

In summary, although all monkeys were completely suppressed by treatment day 6, pituitary response to GnRH was retained. After 3 days post-treatment, although tonic gonadotropins remained suppressed, pituitary release of FSH and particularly LH in response to a GnRH challenge test had increased when compared to the test administered during antagonist treatment. None of the monkeys showed either local or general evidence of histamine release side effects. We conclude that: (1) the GnRH antagonist used in this study is potent and without discernible side effects; (2) pretreatment levels of gonadotopins (particularly FSH) are predictive of the treatment time needed to achieve full pituitary suppression of tonic gonadotropin secretion; (3) normal pituitary response to a GnRH challenge test is retained for several days; and (4) recovery of pituitary secretory capacity in response to a GnRH bolus is manifest before restoration of tonic levels of serum gonadotropins.

This GnRH antagonist, as well as others without significant histamine release effects, deserve consideration for pursuit in clinical trials. The rapidity of action of antagonists of GnRH and their absence of interim stimulatory effects (such as agonists manifest) may allow greater practical utility for both therapeutic and diagnostic applications.

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8

THE REPRODUCTIVE, ENDOCRINE AND PHARMACOLOGICAL EVALUATION OF A NEW LHRH ANTAGONIST

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INTRODUCTION

LHRH agonists suppress the reproductive endocrine system through desensitization and are potentially useful in the treatment of clinical disorders such as uterine fibroids, precocious puberty, endometriosis and prostatic carcinoma. The development of these peptides has progressed in recent years and their clinical efficacy and safety are established. A drawback to the use of these agonists, however, is related to the initial stimulation of the reproductive endocrine system which may exacerbate symptoms of the disease being treated. LHRH antagonists also suppress the reproductive endocrine system but do so by competitively inhibiting binding of LHRH to its receptors and so avoid the initial stimulation which occurs with agonists. Unfortunately, the development of LHRH antagonists was impeded when facial edema was observed in toxicity studies in rats being treated with one of the early antagonists, [N-Ac-D-Nal(2)^{1},D-pF-Phe^{2},D-Trp^{3}, D-Arg^{6}]LHRH (ORF 18260) [1]. Later this peptide was also shown to release histamine [2] and induce cutaneous anaphylaxis [3] which are thought to be the causes of the facial edema which had been observed. Similar reactions were subsequently observed with other LHRH antagonists. These findings raised serious doubt concerning the safety in clinical use of these LHRH antagonists.

Research efforts were then directed towards further modification of the peptide structure of the prototype LHRH antagonists in an attempt to separate the desirable LHRH-blocking property from the undesirable anaphylactoid-like activity. Over forty peptides were screened for anti-ovulatory and anaphylactoid activity in rats, and it was shown that these two activities were not linked [4-6]. These encouraging results suggested that an LHRH antagonist devoid of undesirable anaphylactoid activity could be synthesized. This effort led to the discovery of ORF 21243 ([N-Ac-D-Nal(2)^{1},D-pCl-Phe^{2}, D-Pal(3)^{3},D-Glu(AA)^{6}, D-Ala^{10},]LHRH) as a potent LHRH antagonist with an improved therapeutic ratio relative to that of ORF 19260 [5,7]. Subsequently, a third generation peptide with an even more improved margin of safety was identified. This peptide is ORF

23541 ([N-Ac-D-Nal(2)^{1}, D-pCl-Phe^{2},D-Pal(3)^{3},Lys(Nic)^{5}, D-Lys(Nic)^{6},Lys(iPr)^{8},D-Ala^{10}]LHRH). The evaluation of its LHRH-inhibiting and anaphylactoid activities are described in this chapter.

SUPPRESSION OF GONADOTROPIN RELEASE

ORF 23541 was characterized as an LHRH antagonist in three test systems. First, the ability to inhibit LHRH-induced LH release was examined in dispersed male rat pituitary cells in vitro. ORF 23541 has an IC_{50} of $2x10^{-10}$M in this assay [8].

The antiovulatory activity of ORF 23541 was demonstrated in proestrous rats. The antagonist was administered in the afernoon of proestrus and on the following morning, the oviducts were examined microscopically for ova. ORF 23541 blocked ovulation, with an ED_{50} of 1.45 µg [8].

Finally, the LHRH antagonist activity of ORF 23541 was also evaluated by its ability to suppress gonadotropin levels in cannulated ovariectomized rats. Ovarian steroids provide negative feedback to the pituitary and removal of the ovaries results in an elevation of gonadotropins. ORF 23541, like other LHRH antagonists, blocks this elevation (Fig. 1). LH levels were

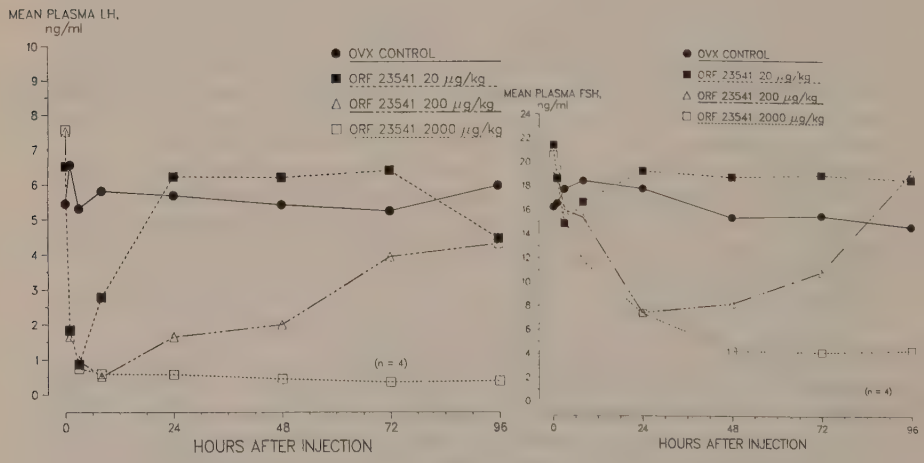

FIGURE 1 The suppression of (A) plasma LH and (B) plasma FSH levels in ovariectomized cannulated rats after a single s.c. injection of ORF 23541. (Reprinted with permission from Life Sci [8])

maximally suppressed within 3 hours after subcutaneous injection of 20, 200 or 2000 µg/kg of ORF 23541 [8]. The recovery was dose-related with a return to castrate levels by 24 or 72 hours after injection with 20 or 200 µg/kg, respectively. LH levels remained suppressed at 96 hours after injection of 2000 µg/kg. FSH levels were similarly affected except that the onset of and recovery from the inhibiting activity of ORF 23541 were delayed as compared to changes in LH levels. The persistent suppression of LH levels 96 hours after injection of 2000 µg/kg of ORF 23541 was different from historical data obtained for ORF 21243 [9]. This prompted us to re-evaluate the two antagonists side by side. The results of this experiment confirmed the long-acting nature of ORF 23541 (Fig. 2). A single injection of 2000 µg/kg of either

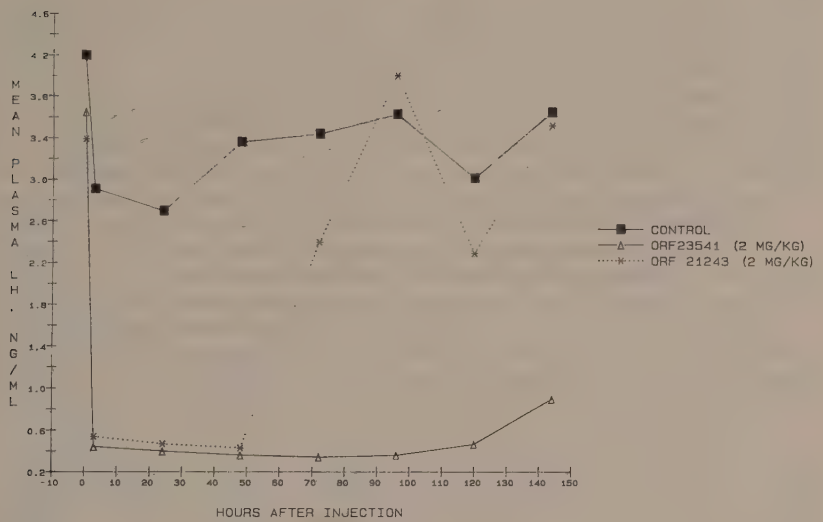

FIGURE 2 The suppression of plasma LH levels in ovariectomized cannulated rats after a single s.c. injection of 2000µg/kg of either ORF 23541 or ORF 21243. (Reprinted with permission from Life Sci [8])

peptide maximally suppressed LH levels within 3 hours [8]. A return to castrate levels occurred at 72 hours after injection of ORF 21243, whereas LH remained suppressed at 96 hours after injection of ORF 23541. Similar long-lasting effects of ORF 23541 were observed in castrated monkeys [10].

CUNTANEOUS ANAPHYLACTOID-LIKE ACTIVITY

The facial edema in rats resulting from administration of ORF 18260 has been characterized by reference to vascular permeability

changes. Those studies suggested that the facial reactions were identical to a cutaneous anaphylactoid-like reaction, possibly as a result of the direct action of the LHRH antagonist on mast cell mediator release. We have used the induction of cutaneous vascular permeability changes (wheal response) in rats and guinea pigs as a predictor of the risk of causing such a reaction clinically in man [11]. In this model, Evan's blue dye is injected intravenously into the tail vein. The dye binds to serum proteins. Increases in vascular permeability can then be visualized as serum proteins, carrying the dye, diffuse into the interstitium and stain connective tissue. Immediately following dye injections, various dilutions of GnRH antagonists were injected intradermally into a shaved section on the back of the animal. Fifteen minutes after the intradermal injection, the animals were killed, the dorsal skin reflected, and the area of wheal was estimated using the mean of the longest perpendicular diameters. Regression analysis was used to estimate the dose producing a 8.75 x 8.75 mm wheal.

Relatively high doses of ORF 23541 were required to cause a wheal response: 10.9 and 13.7µg in rats and guinea pigs, respectively (Table 1). In contrast, much lower doses of ORF 21243 and ORF 18260 caused the same response. The therapeutic

Table 1. Cutaneous Anaphylactoid-Like Activity (Wheal Response) and Therapeutic Ratio of LHRH Antagonists in Rats and Guinea Pigs

Peptide	Species	ED 8.75 x 8.75 µg/wheal (95% F.L.)	Antiovulatory ED_{50}, µg (95% F.L.)	Therapeutic Ratio[a]
LHRH	Rat	ND[b] ---	---	
LHRH	Guinea Pig	ND[b] ---	---	
ORF 23541	Rat	10.9 (5.8-24.7)	1.45 (1.0-1.9)	7.52
ORF 23541	Guinea Pig	13.7 (7.5-32.3)	---	---
ORF 21243	Rat	0.48 (0.20-0.94)	0.50 (0.30-0.55)	0.96
ORF 21243	Guinea Pig	1.75 (1.15-2.82)	---	---
ORF 18260	Rat	0.01 (0.0004-0.05)	0.65 (0.46-0.85)	0.02
ORF 18260	Guinea Pig	0.77 (0.55-1.08)	---	---

[a]Therapeutic ratio = µg (ED anaphylactoid activity)/µg (ED_{50} anti-ovulatory activity)).

[b]The slope of the line for LHRH was not significantly different from zero. An ED wheal was not determined with doses up to 10 µg.

ratio of ORF 23541 in rats (7.52) was much better than that of ORF 21243 (0.96) or ORF 18260 (0.02). These studies suggest an improved margin of safety of ORF 23541. The intradermal administration used in these studies is more provocative than the subcutaneous and intramuscular routes proposed for clinical use. Therefore, this calculated ratio may underestimate the clinical therapeutic index.

CARDIOPULMONARY EVALUATION

Mast cell degranulation with release of mediators including histamine may also affect pulmonary function as these mediators are potent bronchospastic agents. We have evaluated the cardiopulmonary effects of LHRH antagonists in anesthetized dogs and guinea pigs [11]. Guinea pigs were placed in a whole body plethysmograph. A jugular vein and a carotid artery were cannulated for compound administration and for monitoring of blood pressure, respectively. The trachea was also cannulated to allow ventilation at a constant volume via a miniature Starling pump. One differential pressure transducer was used to measure transpleural pressure and tidal volume was monitored by a second. The guinea pigs were treated with succinyl choline to arrest spontaneous respiration. Following a five-minute stabilization period one of the LHRH antagonists was administered i.v. in a volume of 1.0 ml over 30 seconds. In dogs, a femoral artery was cannulated for monitoring blood pressure and a femoral vein was cannulated for administering compounds. A tracheal cannula was inserted and connected to a pneumotachograph for the measurement of air flow. The other side of the pneumotachograph was attached to a respirator for ventilating the animal. An intrapleural cannula was surgically implanted in the 6th intercostal space to monitor intrapleural pressure. The LHRH antagonist was administered i.v. in a volume of 5.0 ml over 30 seconds. In both species, airway resistance and dynamic lung compliance were calculated by a pulmonary computer. Percent change from baseline was monitored over 30 minutes for all parameters and recorded at 1, 3, 5, and 30 minutes after administration.

ORF 23541 did not affect pulmonary function in guinea pigs (Figure 3) or dogs (Figure 4). In contrast ORF 21243 and ORF 18260 did affect pulmonary function. Bronchospasm was observed in dogs after 10mg of either ORF 18260 or ORF 21243, as evidenced by increase in airway resistance and an associated drop in compliance. A severe bronchospasm was observed with 10mg of ORF 21243 in guinea pigs. Relatively high intravenous doses (10mg) were required to observe this effect and a safety factor is demonstrable, i.e. lack of pulmonary effects at a dose of 1mg. However, the finding itself represents a potential risk with these two antagonists. These results suggest that the risk of pulmonary effects in the clinical use of ORF 23541 is very low and that this peptide has an improved safety margin compared to the other two antagonists.

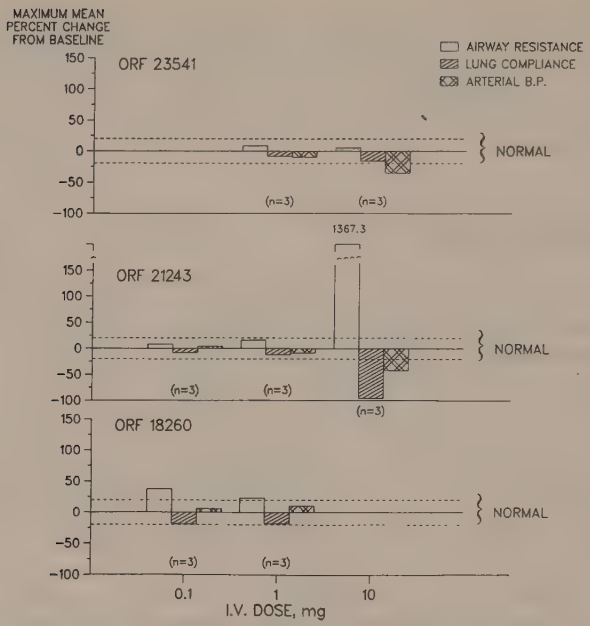

FIGURE 3 Effects of LHRH antagonists on cardiopulmonary function in guinea pigs

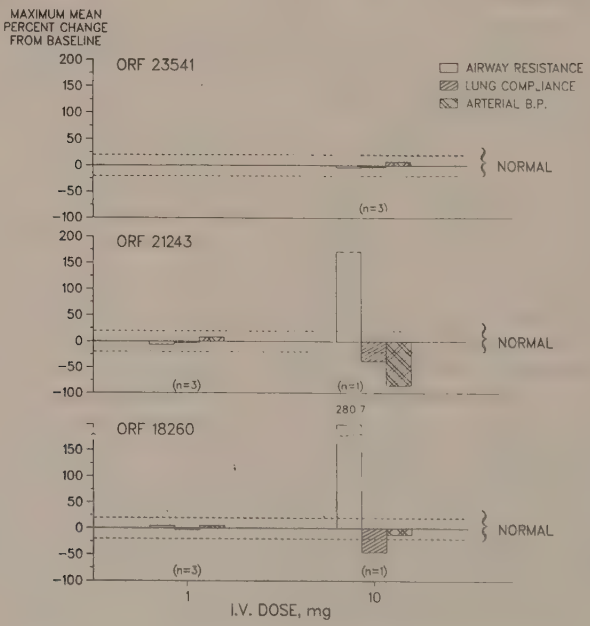

FIGURE 4 Effects of LHRH antagonists on cardiopulmonary function in dogs

A slight decrease in arterial pressure was caused by 10mg of ORF 23541 in guinea pigs, but no such change was seen with this dose in the dog, or with lower doses in the guinea pig.

CONCLUSION

ORF 23541 represents a new generation of potent LHRH antagonists with potentially improved margin of safety. It is hoped that these encouraging findings abate the concern regarding the clinical safety of these peptides and stimulate renewed interest in the development of LHRH antagonists as therapeutic agents.

ACKNOWLEDGEMENTS

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9

PHARMACOKINETICS OF LHRH ANALOGUES

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INTRODUCTION

Peptides are particularly well suited for therapy, because their metabolism differs markedly from conventional drugs. Biotransformation to inactive metabolites and amino acids is rapid, the capacity of peptidase enzymes is high, and enzyme induction has not been observed. LHRH agonists have an important therapeutic role in the control of gonadal steroid secretion in reproductive disorders, and in oncological indications (prostatic carcinoma and premenopausal mammary carcinoma). Their actions on gonadotropins and gonadal steroids are highly selective, with no side effects on the secretion of other hormones or on other organ systems [1-3]. The investigation of peptide pharmacokinetics and metabolism has made rapid progress due to the advances in analytical methodology. In this review, results on buserelin [D-Ser(tBu)^6,Pro^{9}NHEt]LHRH and other LHRH agonists are summarized and discussed with reference to the clinical implications for suitable dose regimens in different disorders.

ORGAN DISTRIBUTION

The organ distribution of isotope-labelled LHRH (gonadorelin) and the LHRH agonist leuprolide ([D-Leu^6,Pro^{9}NHEt]LHRH), nafarelin ([D-Nal(2)^6]LHRH), and buserelin has been investigated in experimental animals. Accumulation of drug is found in the liver, kidney, anterior pituitary and intestine. In studies with 125-I-buserelin in rats, the liver and kidney are the main inactivating organs [4]. High tissue:plasma ratios are reached in these tissues with a maximum about 1 h after i.v. injection [5]. In terms of the percent dose, the liver and kidneys accumulate most of the radioactive drug and metabolites. Radiolabelled metabolites are also excreted in bile. A small fraction of the dose accumulates in the anterior pituitary gland with a time course proportional to the stimulation of LH release after a single dose. The amount of radiolabel excreted in the urine is proportional to the plasma concentration. The urinary peptides can be identified mainly as metabolites [6]. Reports on the organ

distribution of 125-I-buserelin [5], and 3-H-LHRH or 14-C-nafarelin [8] reach similar conclusions on the organs which show accumulation. When 125-I labelled buserelin is administered to the rat, there is a time-dependent increase of 125-I in the thyroid gland, due to the uptake of iodine resulting from metabolic inactivation and deiodination of tyrosine (the site of labelling). No intact peptide is found in the thyroid tissue.The fraction of intact peptide circulating in the serum or plasma decreases with time after injection, thus half-life estimates

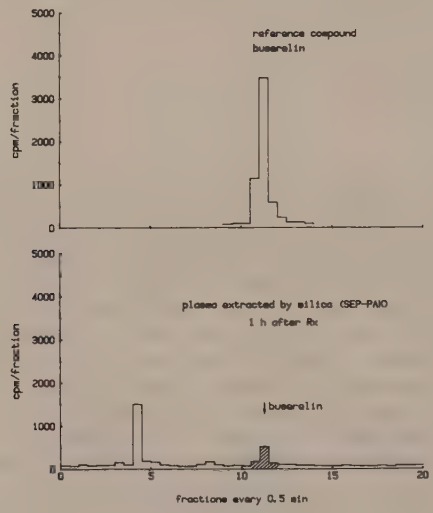

FIGURE 1 Organ distribution study of 125-I-buserelin in male rats. Separation of intact buserelin and metabolites by HPCL. Upper panel: buserelin reference compound, lower panel: plasma sample obtained 1 h after treatment with 2 uCi/1.5ng 125-I-buserelin i.v., extracted by C18-silica (SEP-PAK) and separated on NOVA-PAK C18-column in a reversed phase system.

based on radioactive drug equivalents in the serum or plasma lead to erroneous half-life estimates. We found that in rats treated with 125-I-buserelin metabolism is rapid. The plasma values obtained 1 h after injection consist mainly of metabolites (Fig. 1), when analyzed by thin layer chromatography (TLC) or by high performance liquid chromatography (HPLC). In the HPLC separated plasma extract, 12.6% intact buserelin is identified. The circulating 125-I is not retained by C8-silica extraction (SEP-PAK cartridges). The fraction of intact buserelin in plasma can be increased by saturating the inactivating enzymes by a high dose of an LHRH antagonist administered prior to 125-I-buserelin [24].

With 14-C-nafarelin and 3-H-LHRH, it has also been observed that the fraction of intact peptide in the plasma decreases rapidly after treatment, whereas the total radioactivity circulating in the plasma remains high [7]. Significant differences exist with regard to protein binding. The hydrophobic agonist, nafarelin, is about 80% bound to plasma albumin [9], whereas leuprolide and buserelin have a protein binding of 15-20%, similar to LHRH [8].

METABOLISM

The biotransformation of buserelin to inactive metabolites has been investigated in rats with 125-I-buserelin, isolating the radiolabelled metabolites from liver, kidney, urine and plasma and comparing them with the unlabelled metabolites found in plasma, bile and urine of rats. Results of different studies using identification by TLC or HPLC are in good agreement. One hour after treatment, the livers of rats contain no intact buserelin, but a spectrum of smaller C-terminal metabolites (Fig. 2). In the kidneys, intact buserelin and several C-terminal metabolites are present, one of which is the buserelin [5-9] pentapeptide (Figure 3). This metabolite is also the major product found in the urine. It has also been found in the urine of all species investigated, including the human [2]. Intact buserelin and a variable proportion of the buserelin(2-9)octapeptide are also found in the pituitary gland [4].

Inactivation by enzymes

In contrast to LHRH , which is inactivated by several exo- and endopeptidases, many of the LHRH agonists are relatively resistant to enzyme degradation [5, 9, 10]. Incubation of buserelin with pyroglutamyl-aminopeptidase gives the (2-9)octapeptide from cleavage of the N-terminal bond between pyroglutamic acid and histidine. Both intact buserelin and C-terminal metabolites, e.g. the (2-9)octapeptide can be cleaved by chymotrypsin-like enzymes acting on the Trp^3-Ser^4 and Tyr^5-D-$Ser(tBu)^6$ bonds [11].
In the nonapeptide-ethylamide agonists, inactivation by the post-proline cleaving enzyme is blocked, whereas this enzyme can act on the decapeptide agonists like nafarelin and tryptorelin ([D-Trp^6]LHRH). The azaGly-substitution in goserelin ([D-$Ser(tBu)^6$,$azGly^{10}$]LHRH) should also afford protection. Substitution by D-amino acids in position 6 does not completely inhibit the inactivation by chymotrypsin, because of limited stereoselectivity. The buserelin(5-9)pentapeptide is gradually converted to the (6-9)tetrapeptide, and the nafarelin(5-10) hexapeptide can be converted to the nafarelin (6-10)pentapeptide by chymotrypsin [9]. It is difficult to assess the relative contributions of chemical substitutions to enhanced receptor affinity or to enzyme resistance. In the hydrophobic decapeptide agonists an unprotected Pro^9-glycinamide terminus is compatible

with high potency, suggesting that such agonists may be inaccessible to receptor-associated enzymes after binding.

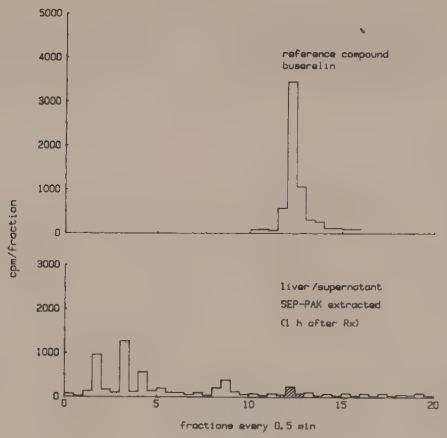

FIGURE 2 Organ distribution study of 125-I-buserelin in male rats. Extraction of peptides from liver homogenate (supernatant fraction) by C18-silica (SEP-PAK) and separation on a NOVA-PAK C18-column in a reversed phase system (28). Intact buserelin indicated by shaded area.

Biliary excretion

From studies with labelled LHRH agonists, a significant fraction of the dose is excreted with the bile and found in the intestine, [7, 9]. After unlabelled buserelin, the only metabolite excreted in the bile of rats was identified as the buserelin (5-9)pentapeptide [12]. A bile:serum ratio of 140 exists for the (5-9) pentapeptide 120 min after s.c. injection of buserelin, indicating that an active secretory mechanism concentrates the (5-9)pentapeptide in the bile. With 14-C-nafarelin, the biliary metabolites identified in the rat [9] were the (5-10)hexapeptide, and smaller amounts of the [5-7)tripeptide, the (1-7)heptapeptide, and the (1-9)nonapeptide. Biliary excretion of buserelin is less than 1% in the dog, indicating that renal elimination may be more important in this species.

Urinary excretion

Immunoreactive LHRH is excreted in the urine of rats and human in small quantities that require concentration by extraction for detection [13]. After administration of equal doses of LHRH and

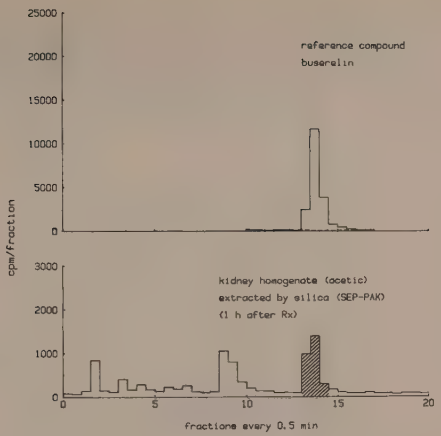

FIGURE 3 Organ distribution study of 125-I-buserelin in male rats. Extraction of peptides from liver homogenate (in 0.1-M acetic acid, supernatant fraction) by C18-silica (SEP-PAK) and separation on a NOVA-PAK C18-column in a reversed phase system (28). The peak in fraction 9 corresponds to the buserelin (5-9) pentapeptide, intact buserelin is found in fraction 14 (shaded area).

buserelin in rats, the fraction of the dose of LHRH in the urine was 0.002%, whereas 25-30% of the buserelin dose was recovered [6]. The daily injection or infusion of 60µg buserelin per rat resulted in a consistent urinary excretion over 14 days. For an analytical identification of the urinary peptide material, we developed a separation by high performance liquid chromatography (HPLC), with specific buserelin radioimmunoassay (RIA) as the detection method [14].

PHARMACOKINETICS

Early studies with synthetic LHRH showed that after an initial rapid distribution phase, this decapeptide has an elimination half-life of 10-12 min [15]. In contrast, highly active LHRH agonists such as leuprolide, nafarelin, goserelin and buserelin are eliminated much more slowly [8,16-19]. The peptides can be measured in the circulation by specific radioimmunoassays [8, 11, 17, 19-21], which detect the two groups of the peptide agonist with a C-terminal Pro^9-glycinamide, and nonapeptide agonists with a C-terminal Pro^9-ethylamide. The results of such measurements should be controlled by chromatography of serum samples to separate intact peptide and biologically inactive metabolites [15]. We have found that extraction on C18-silica cartridges, HPLC for separation, and RIA with specific buserelin

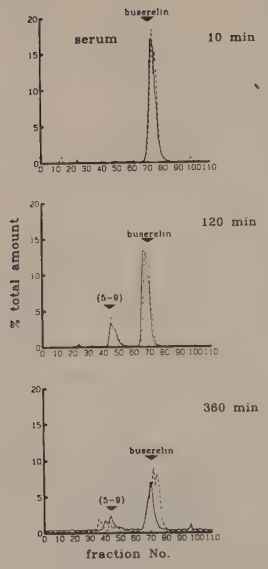

FIGURE 4 Pharmacokinetics of unlabelled buserelin in the human. Patients with endometriosis, injection of buserelin 500µg i.v. Serum samples taken 10, 120 and 360 min after injection. Intact buserelin and metabolites in serum extracts separated by HPLC (28) and measured by specific buserelin RIA. Intact buserelin and the main metabolite, the (5-9) pentapeptide, marked by arrows.

antiserum is a highly selective and sensitive combination detection method . In 6 women treated with an i.v.injection of 500µg of buserelin, the elimination half-life was 51 min. The concentrations of total immunoreactive buserelin measured in unextracted sera were 101.4 ± 33 ng/ml 20 min after injection and 1.12 ± 0.12 ng/ml 360 min after injection. In serum extracts analyzed by reversed-phase HPLC and RIA (Figure 4), intact buserelin was the main component. The main serum metabolite was the inactive buserelin(5-9)pentapeptide. In the urine collected 0-1 h after treatment, 66% intact buserelin and 28% of the buserelin(5-9)pentapeptide were found (Figure 5). In the urine collected between 6-24 h, the ratio of intact buserelin to (5-9)pentapeptide remained similar. The urinary buserelin/ creatinine ratio 0-1 h after treatment was 345 ± 156 µg/g creatinine, and 6-24 h after treatment the concentration was 4.7 µg/g creatinine. These results confirmed that buserelin is eliminated more slowly than LHRH, and remains available to the pituitary receptors for a long period after injection of high doses. After s.c. injection of 1000µg, buserelin the half-life

estimate between 1 and 8 h after injection is 80 min. It is thus clear that high dose injections can saturate pituitary receptors and maintain down-regulation for a 24 h period, as illustrated by the clinical efficacy of single daily high dose injections of leuprolide in prostatic carcinoma.

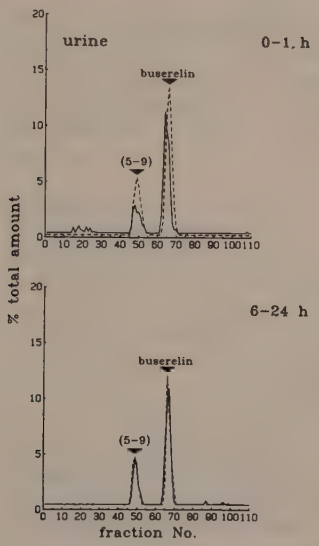

FIGURE 5 Urinary excretion of buserelin and metabolites in patients with endometriosis after treatment with 500µg i.v. Urine samples collected between 0 and 1 h after treatment, and between 6 and 24 h. Intact buserelin and metabolites separated by HPLC (28) and measured by specific buserelin RIA. The ratio of intact buserelin and the main metabolite does not change with time after injection.

Treatment by nasal spray

The urinary excretion of immunoreactive buserelin after treatment with nasal spray is dose-related. Nasal spray medication is a significant improvement for long-term therapy with peptides. The absorption of buserelin in humans is 2.5 to 3.3% of the nasal spray dose [23]. Two bioequivalent formulations of buserelin have been investigated for their LH-releasing activity in comparison with s.c. injections. Single doses of 300 or 400µg nasally give comparable LH-release to 10µg s.c. The serum concentrations after nasal spray of buserelin and nafarelin have been monitored [11, 20, 21, 24]. Peak concentrations of immunoreactive buserelin are reached within 30-60 min. The time course of LH-release induced by the spray is similar to that after an s.c. injection

of buserelin. In the urine, maximum concentrations of immunoreactive buserelin are reached during the first 2 h after nasal treatment, with a time-dependent decline. The effective drug concentration during long-term treatment can be monitored by the daily cumulative excretion, collecting the urine for 24 h during the administration of consecutive doses (Table 1), or by collection for an 8-h period

Table 1. Excretion of immunoreactive buserelin measured by RIA in unextracted urine samples.

Buserelin Dose µg/24 h	Urinary Buserelin µg/24 h
2 x 300 µg	3.1 ± 0.7 (12)
3 x 300 µg	3.8 ± 1.1 (19)
4 x 300 µg	7.0 ± 0.7 (23)
5 x 300 µg	10.3 ± 1.4 (16)
8 x 300 µg	26.0 ± 4.1 (36)

Mean and standard error
( ) No. of determinations

after one nasal spray dose. Due to the limited absorption, the urinary excretion after nasal spray treatment is lower than that reached after buserelin injections [23, 25]. In contraception, a single daily dose of buserelin nasal spray is administered to reduce pituitary responsiveness and prevent the preovulatory LH-surge. In other gynecological indications, 3-4 nasal spray doses of 300µg per day are required to suppress oestrogen secretion and induce a reversible amenorrhoea [6].

Treatment by sustained release

The initial studies on buserelin pharmacokinetics were performed with s.c. infusion via an external minipump. The serum concentrations and urinary excretion of buserelin were closely related to the rate of infusion (Table 2). Since LHRH and buserelin may be inactivated by enzymes in serum at room temperature, handling of the samples under careful refrigeration or the addition of bacitracin 10^{-3} M as an enzyme inhibitor is required to obtain reproducible and consistent results [26]. Buserelin in urine samples is stable when handled repeatedly during assay procedures; freezing and thawing did not affect the analytical result [23].

During the infusion studies, steady state conditions are reached after about 6 h, and the urinary excretion of immunoreactive buserelin (µg/g creatinine) remains constant. The monitoring of clinical studies is therefore considerably facilitated. Urine samples may be obtained at predetermined intervals without having to perform a quantitative collection of urine for 24 h. After treatment with buserelin implants, the

Table 2. Buserelin measured by RIA in unextracted serum and urine samples.

Buserelin Dose µg/24 h	Serum Buserelin ng/ml	Urinary Buserelin µg/g Creatinine
25 µg		1.11 ± 0.55 (3)
50 µg	0.33 ± 0.14 (6)	16.43 ± 2.06 (6)
100 µg	0.38 ± 0.08 (14)	25.51 ± 6.51 (11)
200 µg	0.53 ± 0.05 (45)	34.42 ± 5.95 (20)
300 µg	0.95 ± 0.29 (27)	43.24 ± 12.34 (9)

Mean and standard error
( ) No. of determinations

urinary buserelin excretion (µg/g creatinine) reaches a plateau on day 2-3 after treatment, with a gradual subsequent decline over a period of 4-12 weeks, depending on the dose and formulation [3, 14]. In patients with prostatic carcinoma, buserelin implants containing 6.6 mg peptide in a matrix of polylactic/glycolic acid (75:25) produced urinary excretion rates of 10-15µg buserelin/g creatinine, indicating a daily release rate of 50-75µg buserelin when corrected for an average creatinine excretion of 1.5 g/ 24 h, and a fractional excretion of 30% of the dose in the urine. In several studies, the serum concentrations during implant treatment with a dose of 3.3 mg or 6.6 mg were closely correlated with the urinary excretion rates (Fig 6). It is convenient to monitor the release rate by the buserelin/creatinine ratio in daily morning urine samples.

The spectrum of metabolites identified in the urine of the mouse, rat, dog, monkey and human was similar [2], but different amounts of intact buserelin were present. The fraction of intact buserelin in the urine was well correlated with the dose requirement for suppression for gonadal steroid secretion in each species (Table 3). The mouse, being very insensitive to suppression requires an extremely high daily dose of 25 mg/kg s.c., and almost no intact buserelin is recovered from the urine, the peptide material consisting mainly of the (5-9)pentapeptide (Figure 7). In the rat, which also has a high dose requirement, 23% of intact buserelin was excreted. In the dog, which is readily suppressed by buserelin, the highest fraction of intact buserelin (70%) was found in the urine . In the monkey and human, the fraction of intact buserelin was 45-55%, and the buserelin (59)pentapeptide was the main urinary metabolite as in the other species.

In the human during treatment by buserelin injections infusions, or by biodegradable implants the fraction of the dose of immunoreactive buserelin was 16-30%, depending on the experimental conditions (Figure 8). Of this peptide material, about 50-55% was identified as intact buserelin. The excretion of

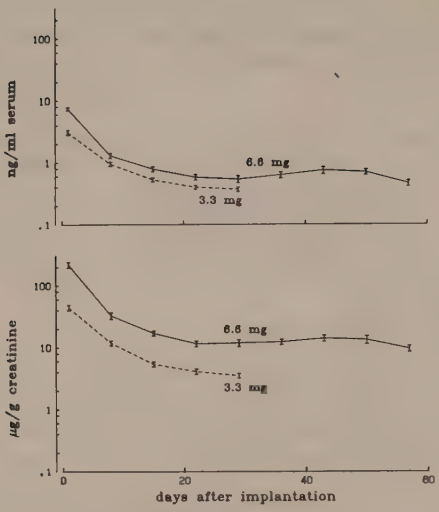

FIGURE 6 Release of buserelin in patients with prostate carcinoma treated with implants containing a dose of 3.3 or 6.6mg peptide in a matrix of polylactide/glycolide 75:25. The release is determined by specific buserelin RIA in unextracted serum samples, and in morning urine samples with a correction for creatinine excretion (28). The buserelin concentrations in serum and urine are well-correlated and dose-dependent.

Table 3. Metabolism of buserelin in different species: dose-requirement for gonadal steroid suppression* and rate of metabolism.

Species	Steroid Suppression	Dose Per Day	% Intact Buserelin in Urine
mouse	testosterone	25 mg/kg	N.D.
rat	testosterone	0.1 mg/kg	22.5
dog	testosterone	2.5 µg/kg	70.2
monkey	oestradiol	10 µg/kg	54.2
human	oestradiol	4 µg/kg	55.1

N.D. nondetectable
*Mouse and rat exhibit partial suppression at the dose indicated, dog, monkey and human are fully suppressed.

buserelin in the milk of nursing mothers was investigated in studies on post-partum contraception [27]. The highest

concentration was 8.8 ng/ml, an a maximum amount of 1-2/µg was calculated as the dose likely to be ingested by infants. There was no charge in urinary LH excretion in these nursing infants, and oral administration of 600µg buserelin had no effect on LH secretion in adult volunteers. It is concluded that buserelin does not enter breast milk in biologically effective concentrations.

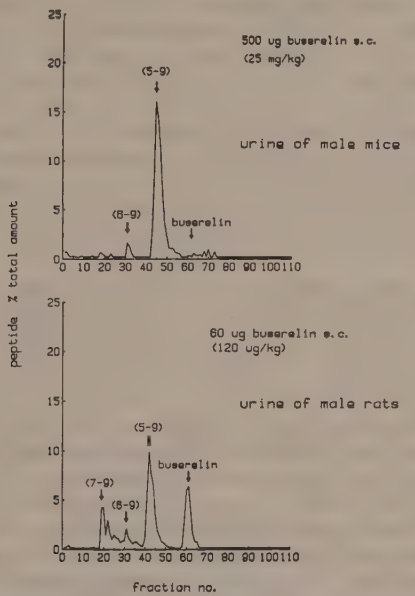

FIGURE 7 Buserelin in urine of mice and rats treated with buserelin injections of 25mg/kg s.c. in mice 0.12mg/kg s.c. in rats. The release is determined by specific buserelin RIA in unextracted urine samples after separation of intact buserelin and C-terminal metabolites by HPCL (28).

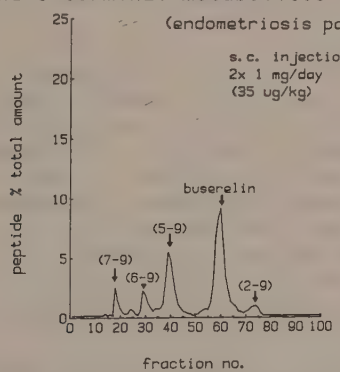

FIGURE 8 Separation of the intact peptide and all C-terminal metabolites excreted in the urine of a patient with endometriosis treated with buserelin injections (35µg/kg s.c. per day). A 25 h urine sample was analyzed by HPLC/RIA [28]. The metabolites identified were as indicated.

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10

MOLECULAR MECHANISMS OF GnRH ACTION ON MAMMARY TUMORS AND UTERINE LEIOMYOMATA

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INTRODUCTION

Carcinoma of the breast is the most common and fatal form of cancer among women in western countries. Many of these tumors are hormone-dependent and are known to be affected by various hormones, such as estrogens, progesterone, corticosteroids, prolactin and insulin [1]. In recent years an additional battery of growth factors which may be released by the tumor cells to act as autocrine growth regulators has been added to the above mentioned list of hormones. The synthesis and secretion of these factors are frequently regulated by the classical hormones, especially estradiol [2]. The autocrine mechanism for the regulation of tumor cell growth, as suggested by Sporn and Todaro [3], is an attractive explanation for the unregulated growth of cancer cells.

The uterine leiomyoma is the most common benign tumor of the uterus and female pelvis. This tumor consists mainly of smooth muscle cells, as well as varying amounts of fibrous connective tissue. The pathogenesis of the myoma is unknown, but estrogens appear to play a role in its growth. The tumor thrives during the years of greatest ovarian activity. However, with the onset of menopause, the decline of ovarian estrogen secretion usually causes the cessation/regression of leiomyoma growth.

The understanding of the basic mechanisms responsible for the growth regulation of mammary tumors and uterine leiomyomata may provide the means for developing therapeutic drugs which would interfere with these mechanisms.

GnRH IN THE TREATMENT OF BREAST CANCER

GnRH analogs have recently been used in several clinical trials for treating breast cancer patients [4]. The rationale for this is illustrated in Fig. 1. According to its known mechanism of action, GnRH, when secreted in a pulsatile fashion from the hypothalamus, stimulates gonadotropin release from the pituitary. However, continuous administration of the peptide causes an

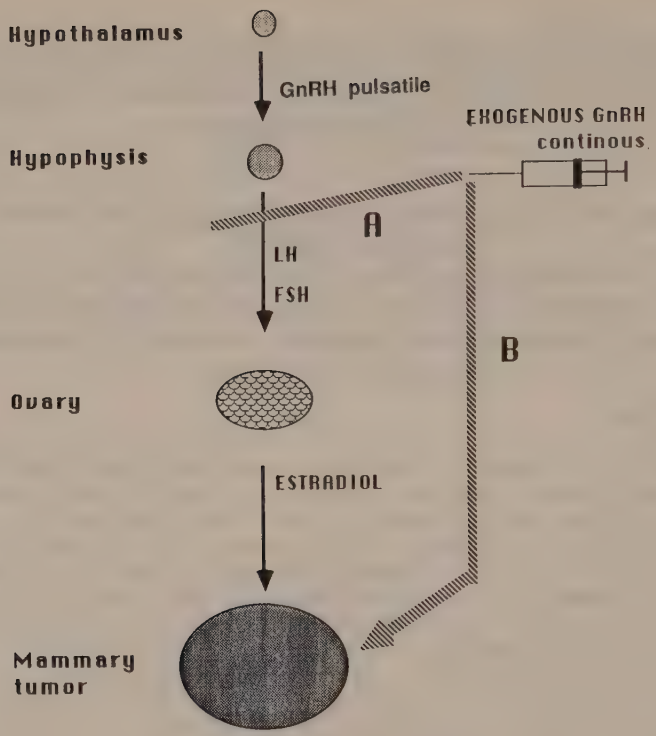

FIGURE 1 Direct and indirect effect of GnRH on mammary tumor growth. GnRH effect on mammary tumors can be explained by the involvement of two mechanisms: A. Indirect effect: Non-pulsatile GnRH administration performs "medical castration" by inhibiing pituitary gonadotropin release, and a decline in ovarian estrogen production. B. Direct interaction of GnRH with the tumor

inhibition of gonadotropin release, as shown by A, which in turn causes inhibition of gonadal steroidogenesis. It was surmised that the low estrogen level was the main reason for the inhibition of mammary tumor growth by GnRH. In this review we will summarize evidence which indicates a direct effect of GnRH analogs on mammary tumors, as illustrated by arrow B. We will discuss primarily the molecular mechanism of the direct action of GnRH in breast cancer, as no direct actions of GnRH on uterine leiomyomata have been reported.

For performing these studies two experimental models were used: (i) the hormone-independent human cell lines in culture - MDA-MB-231, and (ii) the DMBA (7,12-dimethylbenz[a]anthracene)-induced mammary tumor in the rat. The latter serves as an excellent model for the human disease, as its growth rate is

easily manipulated by hormones. Estrogens [5] and prolactin [6] stimulate its growth, while ovariectomy [5], antiestrogens [7] and drugs which decrease plasma prolactin level cause growth arrest or regression of the tumors [8]. We therefore suggested that hormonal transmembrane tranducing mechanisms such as tyrosine kinase activity [8] and the phosphatidyl inositide (PI) cycle [9] may be involved in the growth of these tumors.

SPECIFIC BINDING OF GnRH ANALOGS IN EXTRAPITUITARY TISSUES

Our first aim was to search for possible specific binding sites of GnRH on the DMBA-induced mammary tumor and on uterine leiomyomata membranes. As described in Table 1, specific binding sites for GnRH in different extrapituitary tissues were reported by us and other groups. (For review, see Sandow [18]). The binding sites are characterized by their high specificity but low affinity for various GnRH analogs.

Table 1. Presence of specific binding sites for GnRH in various tissues

Tissue	Reference
Pituitary	10
Breast tumor (biopsy)	11
Breast cancer Human cell lines	12
DMBA-induced mammary tumor in rat	13
Leiomyoma	14
Placenta	15
Testis	16
Ovarian carcinoma	17

GnRH TRANSDUCING MECHANISM

By analogy to the known mechanism of GnRH action in the pituitary [19], we deduce that GnRH may act on mammary tumor membranes via the Phospholipase-C (PL-C) transducing system. As shown in Fig. 2, this transmembranal system includes a receptor which activates the PI specific PL-C affector enzyme, which in turn produces two second messengers - diacyl glycerol and inositol triphosphate.

We developed [20] an in vitro system for measuring phospholipase-C activity in isolated mammary tumor membranes, as can be seen in Fig. 3.

The effect of GnRH on mammary tumor membranes was examined using the GnRH analog [D-Trp^6]GnRH. As shown in Fig. 4, this analog stimulates PL-C activity in a dose-dependent manner. We examined the hormonal effect with two more GnRH analogs (Fig. 5).

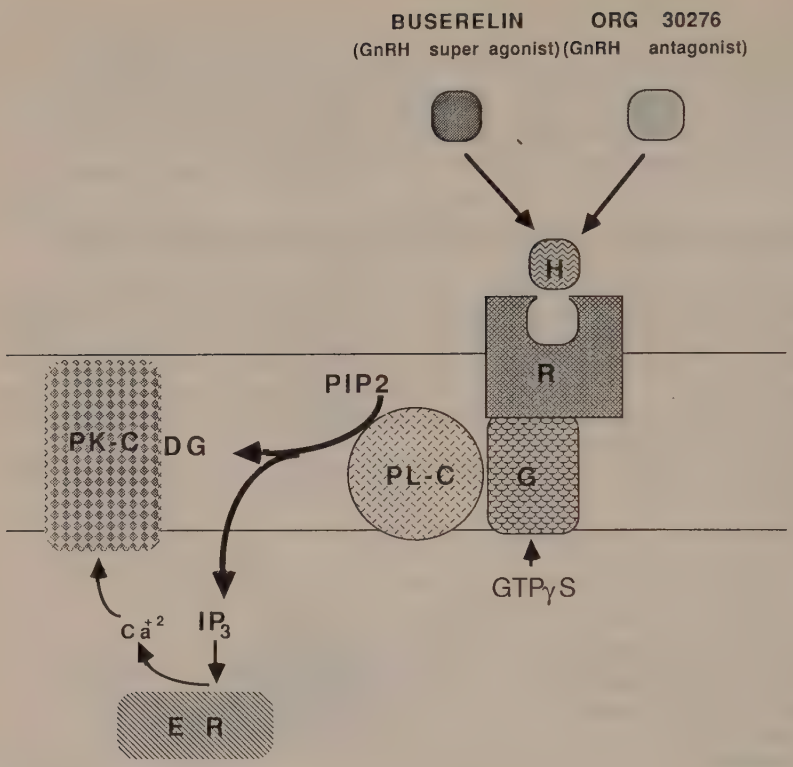

FIGURE 2 Molecular mechanisms for the direct action of GnRH on mammary tumor membranes. GnRH acts on mammary tumor membranes via the Phospholipase-C(PL-C) transducing system. This transmembranal system includes a receptor (R) which activates the phosphoinositide-specific PL-C affector enzyme, which in turn produces two second messengers - diacyl glycerol (DG) and inositol tris phosphate (IP_3) from phosphatidyl inositol diphosphate (PIP_2). IP_3 releases Ca^{2+} from the endoplasmic reticulum (ER), the cation activates protein kinase-C (PK-C) together with DG. G-binding proteins (G) are modulators of this transducing mechanism as shown in experiments with $GTP_\gamma S$, an unhydrolizable analog of GTP.

The stimulatory effect of buserelin [D-Ser(Bu^t)^6,Pro^9 NHEt]GnRH on PL-C activity is greater than that of [D-Trp^6] GnRH. The effect of 10^{-7} M [D-Trp^6]GnRH was completely suppressed in the presence of 10^{-4} M of the GnRH antagonist ORG-30276 ([N-Ac-D-pCl-Phe^1,D-pCl-Phe^2,D-Trp^3, D-Arg^6,D-Ala^{10}]GnRH).

The binding of GnRH and its stimulation of PL-C activity in mammary tumor membranes may trigger the mechanism involved in the direct effect of the decapeptide upon mammary tumor growth.

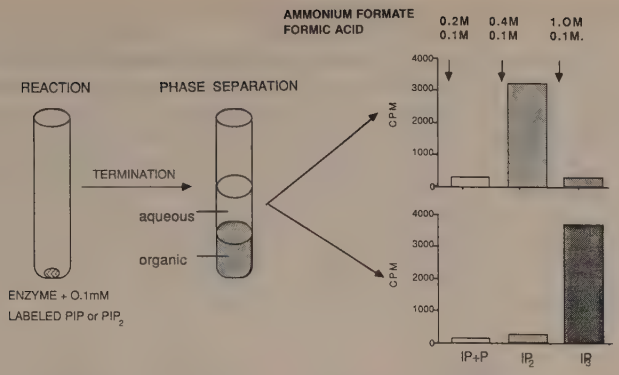

FIGURE 3 Phospholipase-C assay protocol. Isolated membranes from different tissues are incubated in the presence of labelled PIP_2. The reaction is stopped with an acidified chloroform methanol mixture. Separation to two phases is achieved by the addition of water. The upper aqueous phase is applied to Dowex 1+8 columns, and the inositol phosphates are separated by three sequential washes with increasing concentrations of ammonium formate.

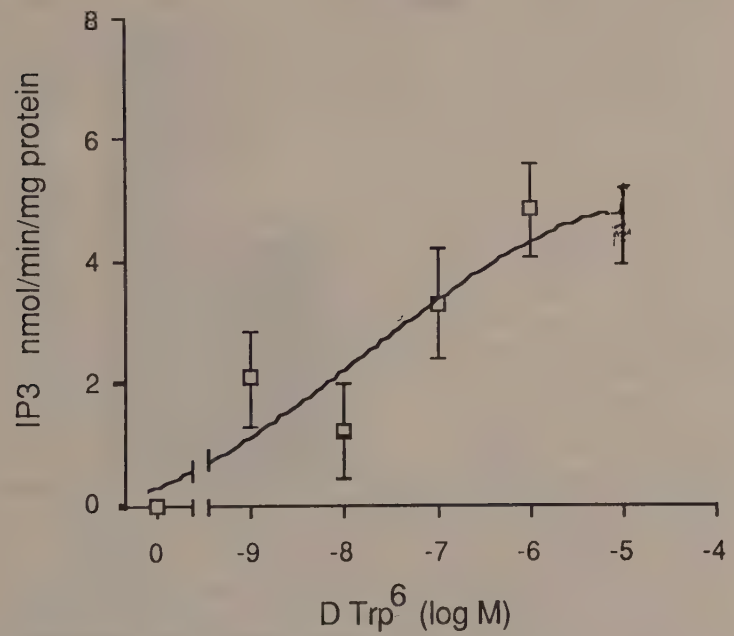

FIGURE 4 Stimulation of membranal PL-C activity by [D-Trp^6] GnRH. IP_3 produced in control incubations (without hormone) was subtracted from all experiments.

Would these basic findings indicate that GnRH may be used in the therapy of breast cancer? The answer is not simple, as is evident from the low rate of response in several clinical studies reported so far [4,21]. One of the explanations for the partial success might be that insufficient emphasis was placed on the direct effect of GnRH analogs on the mammary tumor. This direct effect may be involved in the responses noted in clinical studies performed on postmenopausal breast cancer patients [21]. We have therefore studied the direct effect of buserelin and ORG 30276 on human mammary tumor cell line proliferation.

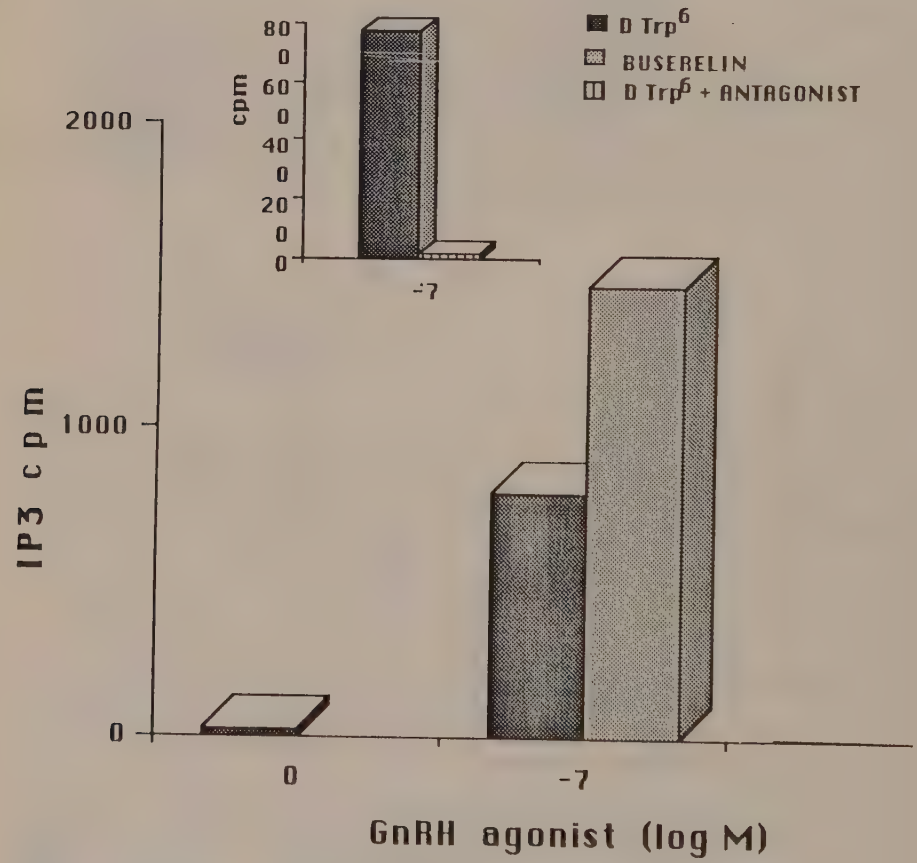

FIGURE 5 Effect of GnRH agonist and antagonist on membranal PL-C activity. The effect of ORG 30276 (10^{-4} M) shown in the insert was assayed in the presence of 10^{-7} M [D-Trp^6]GnRH.

As depicted in Fig. 6, buserelin did not affect thymidine incorporation into the cells. The GnRH antagonist, however, significantly inhibited this incorporation. These results suggest that GnRH antagonists may directly affect mammary tumor cell proliferation. The fact that the GnRH antagonist also inhibits PL-C activity as described above may be relevant to the effect on cell proliferation. However, more studies are needed to clarify this point.

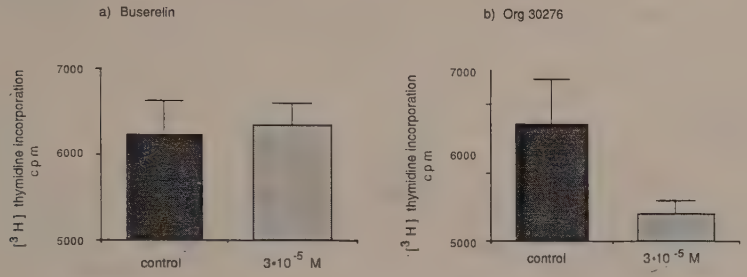

FIGURE 6 Effect of buserelin and ORG 30276 on the proliferation of MDA-MB-231 breast cancer cells in culture. Cells were plated on 96 plating flasks. After 24 hours the cells were incubated either in the presence or the absence of the agonist or antagonist for 24 additional hours before being incubated with labelled thymidine for 3 hours.

FUTURE PROSPECTS

One probably cannot expect to find the same inhibitory effect on mammary tumor growth by GnRH agonist and antagonist as found in the pituitary system in gonadotropin release studies. The receptor(s) for GnRH analogs in mammary tumors may recognize different peptides from those developed for the pituitary system. Consequently, for more effective mammary tumor treatment, the analogs should be screened in assays that will emphasize their direct effect on mammary tumors.

The first screening stage can be performed in the tube (in vitro) by measuring GnRH analog binding and modulation of PL-C activity. Membranes from experimental tumor models or from patients' biopsies will be used. The selected analogs will be tested for their effect on the proliferation of mammary tumor cell lines in culture (second stage). In the third stage, tests on animal models, such as the DMBA-induced mammary tumor in the rat, will be performed before the final clinical trials.

This procedure, which is obviously more economical, can be employed in the selection of the optimal GnRH analog, from among the many available, for the treatment of breast cancer patients.

ACKNOWLEDGEMENTS

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11
KINETICS OF SUSTAINED RELEASE DELIVERY SYSTEMS

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INTRODUCTION

Depot formulations of contraceptive steroids, generally in the form of subcutaneous implants of injectable particles, have been known for more than twenty years, [1, 2] but the work on sustained release of hormonal peptides was started relatively recently. Polypeptides need special delivery systems to circumvent the general lack of oral activity and the short duration of action once they are released in the systemic circulation.

The discovery of the decapeptide LHRH , presented a particular therapeutic challenge for several reasons. Stimulation is obtained when LHRH is given at low doses and intermittently (or in pulsatile fashion), but the opposite (antagonistic) effect prevails when this peptide is administered continuously. Therefore, when optimal antagonistic effect is desired, to suppress tumor growth for instance, the LHRH agonist has to be administered with a specially designed sustained release delivery system.

A group under this author's direction [3] was probably the first, in 1974, to point out the usefulness of microencapsulation for intramuscular sustained delivery of LHRH. The first description of microencapsulation of an LHRH agonist (nafarelin) using dl lactide-coglycolide (PLGA) was in 1984 [4], this was subsequently refined and characterized [5-8]. The process was also applied to tryptorelin [9]. Researchers from ICI described the use of a solid, implantable PLGA formulation with goserelin [10, 11]. IPSEN-Biotech published a pharmacokinetic study on one such formulation with tryptorelin (D-Trp^6-LHRH) as the active principle [12]. More information on controlled release formulations of LHRH agonists and antagonists has been presented at this Symposium.

KINETICS OF RELEASE

The release pattern of the LHRH analogue is a function of the physico-chemical properties of the peptide as well as of the polymer. It has been recognized that essentially two mechanisms of release operate in sequence: 1) diffusion of the active principle through the polymeric matrix of which the delivery systems are made and 2) hydrolytic dissolution of the polymer with time, which frees additional active principle. An overlapping phase may exist between the two mechanisms. As of today, two LHRH agonists (tryptorelin and goserelin) are commercially available in a depot, slow release formulation and at least three more (nafarelin, buserelin and leuprolide) are under active development.

From my analysis of the published data, bell-shaped patterns of release are obtained as measured by plasma levels of peptide. Two general patterns are thus obtained: a symmetrical curve as in Fig. 1b, with plasma levels increasing to a peak at several days after the injection and a non-symmetrical one (Fig. 1a), characterized by a "burst effect" resulting in a peak within day one of the injection with a secondary lower peak several days later. None of these patterns follows zero-order kinetics, a fact which, at least for LHRH agonists as opposed to antagonists, may have therapeutic significance.

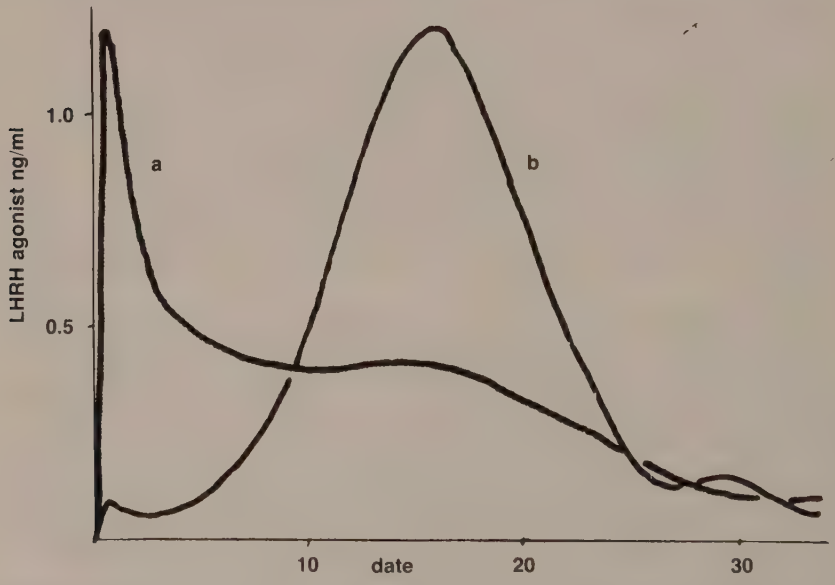

FIGURE 1 Patterns of release of LHRH agonists from sustained release formulations

Sustained release patterns of nafarelin acetate [7] and goserelin [8] are similar to the one depicted in pattern "b" (Fig. 1). On the contrary, tryptorelin [12] leuprolide [14] and

buserelin [15] follow pattern "a" with the early peak due to the burst effect.

Whether it is by pattern a or b, all these formulations have achieved a sustained release of the active principle and have therefore fulfilled one of the requirements for optimal antagonistic activity. The question however should be asked, whether the different release patterns reflect any clinically appreciable difference which would allow a distinction and a preference between them.

Let us examine carefully the evidence:

1) It has been known for some time from kinetic studies and confirmed by recent experiments [16] that the LHRH induced desensitization of rat pituitary cells is dose and time dependent. In other words the gonadotrophin release from the pituitary can be down-regulated by continuous exposure to LHRH. Higher doses result in a more rapid down-regulation, whereas preincubation with lower doses requires a longer period of pretreatment with the releasing factor to achieve the same effect.
2) Animal experiments conducted in male dogs [17] have shown a positive correlation between the dose of tryptorelin released on day 1 and the rate of inhibition of testosterone.
3) Experiments in normal volunteers receiving controlled release injectable preparations loaded with different concentrations (2, 4, 7%) of nafarelin have shown [18] that serum testosterone levels were suppressed earlier with the higher load (7%) which also gave an earlier peak of the agonist.
4) In patients treated with varying doses of sustained release goserelin [11] it was noted that the lowest dose (0.9mg) resulted in the longest time (up to 44 days) to achieve castrate levels of testosterone, a fact which prompted cessation of randomisation and a transfer of the patients to a higher dose (3.6mg).

These facts lead us to postulate that high initial dose and continuous administration is preferable to a gradual increase in dose and continuous administration at least for those patients who are starting treatment and are therefore at some risk of flare-up episodes due to the initial hormonal stimulation.

The "burst-effect" of pattern "a" is then the equivalent of a loading dose frequently given therapeutically when a fast initial effect is desirable.

Obviously only a careful analysis of the decrease rates of testosterone, estradiol and gonadotrophins as related to the release kinetics of various preparations of LHRH agonists will confirm our postulate.

In summary, newer delivery systems of the kind discussed here not only ensure better patient compliance, but represent an optimization of therapeutic effects in terms of safety and efficacy.

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12

FORMULATIONS OF GnRH ANALOGUES FOR THERAPEUTIC USE

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INTRODUCTION

Formulation of drugs rarely elicits much interest amongst bioscientists and clinicians although it is well recognised that the properties of any formulation can have a considerable impact on the successful exploitation of a pharmaceutical agent. Polypeptides usually show low oral potency because their molecular weights are too high to allow effective absorption across the intestinal mucosa and they are rapidly broken down by proteolytic enzymes in gastrointestinal fluids. This is certainly true for luteinising hormone releasing hormone (LHRH) analogues: for example, 'Zoladex' ([D-Ser(tBu)^{6},azGly^{10}])LHRH; goserelin; ICI 118,630), which is around 2000-fold less potent by the oral route than parenterally [1]. For this reason, the parenteral route has been preferred. This paper aims to review the formulations of LHRH analogues which have been used experimentally and clinically and to discuss their advantages and disadvantages.

AQUEOUS SOLUTIONS

Simple aqueous formulations are available for LHRH, buserelin ([D-Ser(tBu)^{6},Pro^{9}NHEt]LHRH), leuprorelin (leuprolide; [D-Leu^{6},Pro^{9}NHEt]LHRH), tryptorelin (decapeptyl; [D-Trp^{6}]LHRH) and nafarelin ([D-Nal(2)^{6}]LHRH). The drugs are usually administered intravenously, subcutaneously or intramuscularly. Aqueous formulations of LHRH and analogues are appropriate for acute administration, where the objective is to elicit gonadotrophin secretion; for example, in the assessment of pituitary function. Aqueous formulations are also currently preferred to use in the treatment of male and female infertility by pulsatile injection from programmed minipumps. However, data in farm animals [2] suggest that continuous infusion of very small doses of LHRH will stimulate ovulation during anoestrus. If these data are confirmed in patients with infertility then perhaps a constant infusion or depot preparation which releases tiny amounts of LHRH or analogues will also be of value.

To achieve pituitary desensitisation and the consequent castration-like effect needed for the treatment of malignant and benign sex hormone-responsive diseases, aqueous injections of LHRH analogues need to be administered one or more times daily.

Although there are reports of the successful treatment of prostate cancer using daily injections of leuprolide [3-4], such formulations are inconvenient to administer and are likely to give compliance problems if self-administered by the patient. For this reason alternative delivery systems and routes of administration have been widely sought.

BUCCAL, RECTAL, INTRAVAGINAL AND PERCUTANEOUS ADMINISTRATION

Apart from im, sc and iv injection, other routes of administration are possible for LHRH analogues; these include intravaginal [5-8], buccal [9] and rectal [10]. Generally these alternative routes of administration are associated with a very low and variable bioavailability and none offers wholly satisfactory solutions to the problems of peptide administration. In the authors' view oral, buccal and rectal administration, even with the use of absorption enhancers or surfactants, will always pose severe problems either in terms of bioavailability of drug or frequency of administration. None of these routes is compatible with genuine long term sustained release of peptide over weeks or months following a single administration.

The transdermal route is suited to the use of long-acting formulations and indeed it has been claimed that polypeptides can be administered percutaneously [11]. It seems unlikely, however, that large molecules such as polypeptides could ever be transported across unbroken and undamaged skin.

INTRANASAL DELIVERY

Apart from simple parenteral administration, the intranasal route has been the most widely studied. Buserelin is marketed as a nasal spray and a similar formulation of nafarelin is being developed [12]. Delivery of an aqueous formulation of an LHRH analogue into the nose is usually by means of a metered dose spray pump [13]. The absorption of LHRH analogues varies between 1 and 10% when delivered in this way [13] which makes accurate dosing difficult.

It has been claimed that the absorption of drug following nasal delivery may also be influenced by rhinitis, local pathological changes in the nasal cavity, and colds [13]. However recent data on intranasal dosing of buserelin shows that experimentally-induced rhinitis has no effect on drug absorption [14]. Although effective and widely used, nasal spray formulations give a number of problems in addition to poor and variable absorption of the drug. The need for multiple daily drug administration inevitably impairs compliance with the treatment

and may, indeed, be the reason why nasal spray therapy with buserelin was less effective than daily parenteral injection [15].

The presence of a preservative in most nasal formulations is one of the most frequent causes of nasal iritations and allergic rhinitis in patients on long term nasal spray therapy according to Harris [13].

For these reasons, we have preferred the parenteral route of administration whih ensures essentially complete bioavailability of compound and have taken the view that the most practical and effective formulation for the administration LHRH analogues is a depot, either as a solid sub-dermal formulation or an injectable suspension, that provides for continuous, sustained release over many weeks or even months.

DEPOT FORMULATIONS

Depot formulations of 'Zoladex', tryptorelin, buserelin and nafarelin have been described [16-19], all of which appear to be based on dispersion of the drugs in co-polymers of lactic and glycolic acids.

These polymers have been selected because long experience shows that they are biocompatible and inert in the physiological environment and degrade to toxicologically acceptable fragments [20]. 'Zoladex' and buserelin are in the form of solid depots for sc injection. Tryptorelin and nafarelin have been studied as powdered solids which have to be dispersed in a solution before administration. The administration at no more than monthly intervals means that medical or paramedical staff can give the injections which should ensure improved compliance.

BIOASSAYS OF RELEASE

Since we are most familiar with the formulation of 'Zoladex' the remainder of this paper will describe the development and properties of 'Zoladex' depots. Continuous release of 'Zoladex' in vivo was measured qualitatively by the biological effect elicited in adult female rats showing regular oestrous cycles. Normally, these rats have an oestrous cycle of 4 days and the occurrence of oestrus is indicated by the presence of cornified cells in vaginal smears. In rats given sub-dermal depots of 'Zoladex', release of drug at an effective rate causes a fall in circulating oestrogens, which in turn leads to a suppression of oestrus and absence of cornified vaginal smears. Rats, therefore, show an extended period of dioestrus.

For amorphous homo- and co-polymers of approximately the same high molecular weight, increasing lactide concentration results in slower degradation. This is refelected in the biological effect elicited in rats using sub-dermal depots containing small amounts of drug in high molecular weight carriers (Fig. 1).

When administered to female rats some drug is released initially as judged by biological response. There then follows a period during which drug is either not released at all or is released at an ineffective rate and so the rats return to oestrus. At some later time point release recommences, the biological response returns and continues until the depot is fully depleted of drug. For polymers of similar molecular weight and

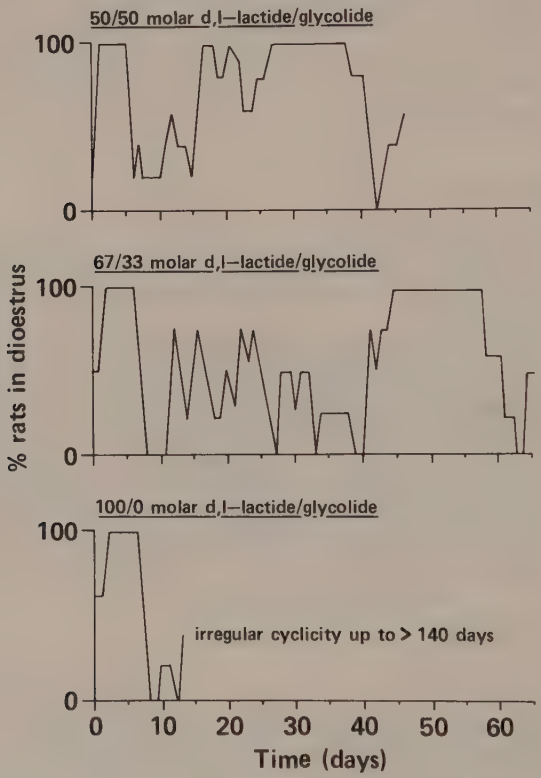

FIGURE 1 Effects of treatment with 100μg 'Zoladex' using subcutaneous depots containing 3$ w/w drug on oestrous cycles in regularly cycling female rats

distribution it can be seen that the interval between the two phases of release is shortest for the most rapidly degradable polymer. The mechanism of release has been extensively reviewed [20].

When these two phases of release do not overlap discontinuous release is observed (Fig. 2A). However, by controlling the properties of the polymer, the initial phase of release can be

made to overlap with the second phase and depots can be defined which give continuous release over not less than 28 days (Fig. 2B).

Depot formulations, which are administered by subcutaneous injection and which release over at least 28 days, have been developed for clinical use. The depots are based on a poly (d,l-lactide-co-glycolide) matrix in which 'Zoladex' (3.6 mg) is uniformly dispersed. The depot is in the form of a cylindrical rod 11 x 1.1 mm.

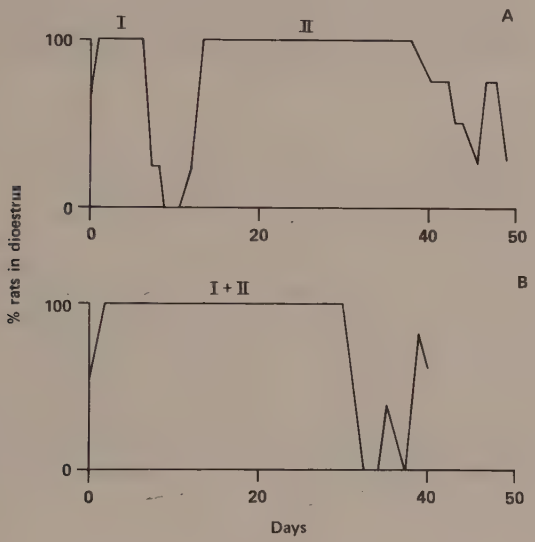

FIGURE 2 Effect of subdermal depots containing 300µg Zoladex on estrous cycling in rats. Depots containing (A) 3% w/w drug in high molecular weight polymer, (B) 20% w/w drug in low molecular weight polymer. I, initial release due to surface leaching; II, degradation induced release

Although the primary objective was to produce a formulation of the drug which was more convenient to administer and which would secure improved compliance, these depot formulations also appear to have improved efficacy. This was demonstrated in rats which were given a single subcutaneous bolus dose of 50 µg 'Zoladex' after either pretreatment with daily subcutaneous injections of saline or 50 µg 'Zoladex' for 6 weeks or, alternatively, at the start of the experiment and at 4 weeks, a single subcutaneous depot, calculated to release a dose of 'Zoladex' equivalent to 50 µg daily. Rats were killed at various times after the final treatment and serum LH was determined by radioimmunoassay in blood collected from the dorsal aorta. The results are shown in Fig. 3.

As expected, the bolus dose of 'Zoladex' elicited a massive secretion of LH in the rats pre-treated with saline. In spite of pre-treatment for 6 weeks with 50 µg 'Zoladex' daily the bolus dose of the drug still caused a substantial release of LH, although a clear degree of desensitisation of the pituitary gland occured. In contrast, there was negligible LH secretion in response to the bolus injection in rats pre-treated with the 'Zoladex' depot, indicating an improved level of pituitary desensitisation with this formulation.

Studies in the pigtailed monkey have also shown that the sc administration of a depot containing 'Zoladex' is more effective in suppressing serum testosterone than daily sc injections of the drug [21]. Recently, two groups have shown that a depot

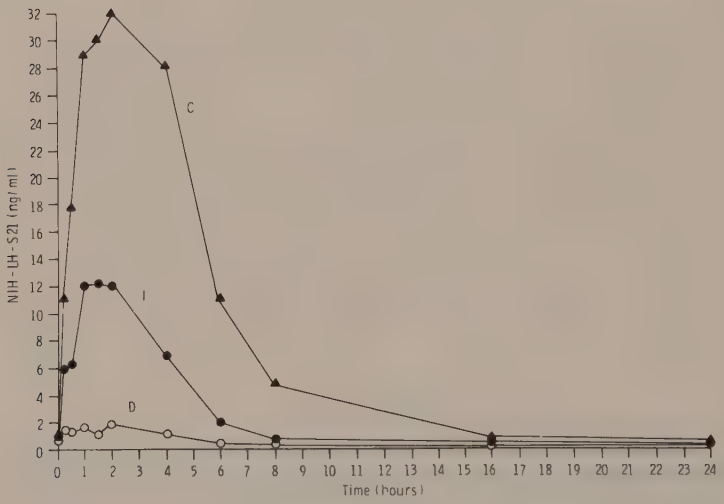

FIGURE 3 Serum LH release in response to a bolus dose of 50µg 'Zoladex' in rats pretreated with saline (C) or 50µg 'Zoladex' (I) daily for 6 weeks. The response after treatment at the start of the experiment and at 4 weeks with single subcutaneous depots, calculated to release 50µg 'Zoladex' daily, is also shown. Serum LH was measured by radioimmunoassay and the values given are the means of 5 samples at each time point

containing 3.6 mg is more effective at suppressing serum testosterone in patients with prostate cancer than daily sc

injections of 250 or 500 µg 'Zoladex' in an aqueous formulation [22,23].

BREAST CANCER

The efficacy of this depot formulation of 'Zoladex' was tested in two sex hormone-responsive tumor models. The first was the dimethyl-benzanthracene (DMBA)-induced rat mammary carcinoma [24] which is known to be dependent on both oestrogen and prolactin [25]. A single sc depot containing 500 µg 'Zoladex' caused an inhibition of oestrogen secretion, the disappearance of cornified cells from vaginal smears and regression of DMBA-induced mammary tumours.

Half of the tumours present at the start of the experiment were not palpable at 28 days but all save one of them reappeared between 40 and 60 days as the single 'Zoladex' depot became exhausted. In contrast, the DMBA-induced mammary tumours increased in size by more than 50% in control animals given a placebo depot.

If single depots of the drug were given sc at weeks 0, 4 and 8 of the study, the regression was more impressive. No tumour was palpable at week 11.

Again, by week 16 regrowth of the tumours occurred because treatment stopped at week 8. By week 20, the tumours had re-attained pre-treatment size.

When given at days 30, 58 and 86 after administration of the carcinogen, single depots containing 500 µg 'Zoladex' delayed the appearance of tumours for a period of around 100 days. That is, the expected duration of action of such a treatment regimen (Fig. 4).

When given at 28 day intervals, starting on day 30 after administrtion of the carcinogen, single sc depots of 'Zoladex' caused a more profound inhibition of tumour appearance and only 9 out of 21 rats had mammary tumours at the end of the study on day 450. Those tumours which were found did not regress following ovariectomy and so were classified as non-hormone responsive. Tt is concluded from this study, which approximates the clinical adjuvant therapy setting, that 'Zoladex' may be of benefit as a treatment for primary breast cancer post-mastectomy in pre-menopausal women.

The second tumour we have used is the Dunning R3327H rat transplantable prostate adenocarcinoma, which is androgen responsive and has been used extensively as a model for the human disease [26].

Single subcutaneous depots containing 1 mg 'Zoladex' given every 28 days to rats bearing Dunning R3327H prostate tumours implanted on each flank caused a marked inhibition of tumour growth indistinguishable from that in surgically castrated rats (Fig. 5). Twenty-one days after the eighth depot was given, the rats were killed and the weights of the sex organs and serum hormone concentrations measured by radioimmunoassay (Table 1).

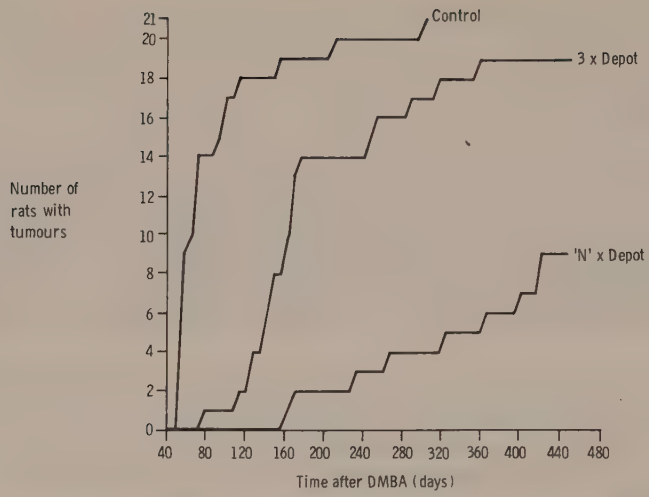

FIGURE 4 Effect of depots containing 500µg 'Zoladex' on the appearance of mammary tumours induced in rats by DMBA. The number of rats with tumours in groups given placebo depots (Control), single depots at days 30, 58 and 86 (3 x Depot), or single depots every 28 days starting at day 30 ('N' x Depot) are shown. Each group comprised 21 rats

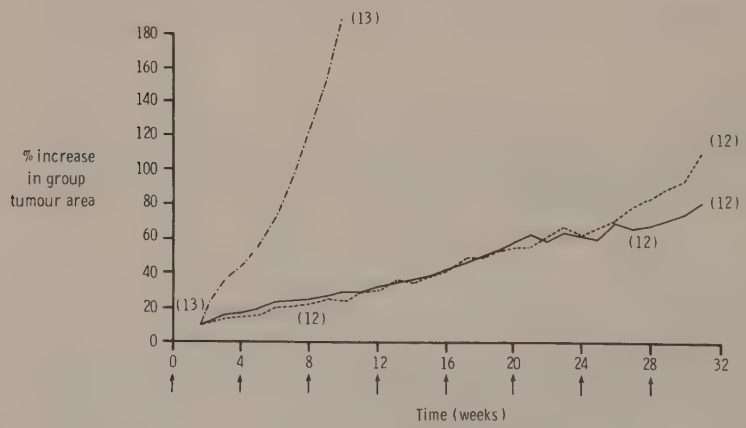

FIGURE 5 Effect of single subcutaneous depots containing 1mg 'Zoladex' on the growth of Dunning R3327H transplantable rat prostate tumours. The depots were given every 4 weeks as shown by the arrows. The control group (- -) had 13 animals and 'Zoladex'-treated (- - -) and surgically castrated (---) groups 12 animals each as shown by the figures in parentheses

Table I: Sex organ weights and serum hormone concentrations in "Zoladex"-treated, and surgically castrated rats bearing Dunning R3327H prostate tumours. Control values for rats of a similar age and weight are shown for comparison.

Group	Tissue Weight (mg)			Sera Hormone Levels (ng/ml)			
	Testes	Ventral Prostrate	Seminal Vesicle	LH	FSH	Prolactin	Testosterone
Zolad	367	21	54	<0.2	175	63	<0.3
Castrate	-	20	54	13	1413	61	<0.3
Intact	3500	250	350	2	400	30	3

Testes weights were about 10% those of control rats of a similar age and weight and showed atrophic histological changes. Ventral prostate gland and seminal vesicle weights were identical to those in the surgically castrated group and, histologically, were also completely atrophic.

Serum LH and testosterone were undetectable in the group given 'Zoladex' depot and serum FSH was decreased by 60-70%. Serum prolactin doubled in rats given 'Zoladex', as it did in surgically castrated animals, probably as a consequence of androgen withdrawal. This contrasts with the effect of 'Zoladex' in female rats where there was a significant reduction in serum prolactin following oestrogen withdrawal [27].

Because of interest in the concept of 'total androgen withdrawal' persuasively supported by Labrie and co-workers [28], a study of the effects of 'Zoladex' depot and a new antiandrogen, ICI 176,334 [29], given alone and in combination, were evaluated. The results (Fig. 6) clearly show that both 'Zoladex' depot and ICI 176,334 are effective at limiting tumour growth but that the combination offers no advantage over monotherapy with 'Zoladex'.

This finding is in agreement with studies [30,31] which showed that antiandrogen failed to improve the effect of surgical castration in rats with Dunning tumours. It is possible that the rat secretes less androgen from the adrenal gland than man and that the rat may, therefore, not be the most suitable species in which to test the hypothesis. The results of the extensive, controlled, comparative clinical studies now being undertaken will, hopefully, soon answer this question.

At present, asymptomatic patients with prostate cancer usually remain untreated probably because of the unacceptability of the most widely used therapy, castration. This policy should be reconsidered now that LHRH agonists and some newer antiandrogens have been introduced. It is important to know whether treatment early in the course of the disease reduces tumour growth rate and

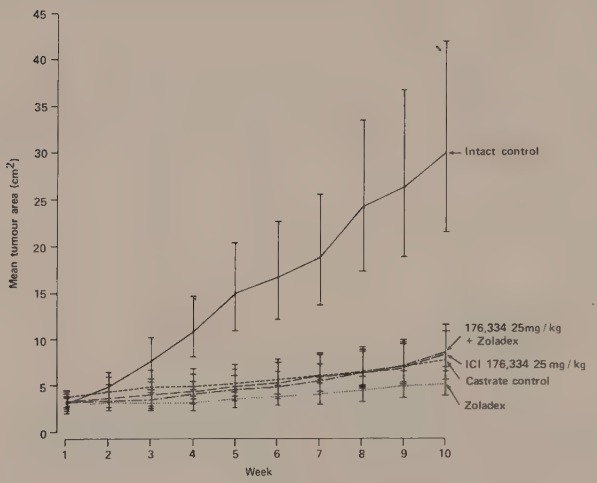

FIGURE 6 Effect of single s.c. 1mg 'Zoladex' depots given every 28 days, daily oral administration of 25mg ICI 176,334/kg and the combination of both treatments on the growth of Dunning prostate tumours. The values shown are means ± SEM; n=8

improves survival. Consequently, a study was made of the growth of Dunning tumours and the survival of rats given single 1 mg 'Zoladex' depots every 28 days starting either 28 days after tumour implantation, when most tumours are not palpable, or when tumours had reached a size of 2 cm^2. Tumour growth was measured at weekly intervals and survival was assessed. In this experiment rats were killed, for ethical reasons, on the week the tumour burden exceeded 20 cm^2. The results shown in Figs. 7 and 8 demonstrate that both treatment schedules reduce tumour growth rate and increase survival but that an early start of treatment produces the best result.

This finding is in accord with the work of Schally and Redding [32] who showed that combination of an LHRH agonist with cytotoxic chemotherapy produced more impressive responses and increases in survival if started early post-transplantation of Dunning tumours. These results argue strongly that consideration should now be given to the treatment of patients with early stages of prostate cancer. It is hoped that clinical trials will soon confirm the animal data.

The promising results achieved in animal studies with 'Zoladex' therapy have now been substantiated in clinical trials in men suffering from prostatic carcinoma and in premenopausal women with advanced breast cancer [33].

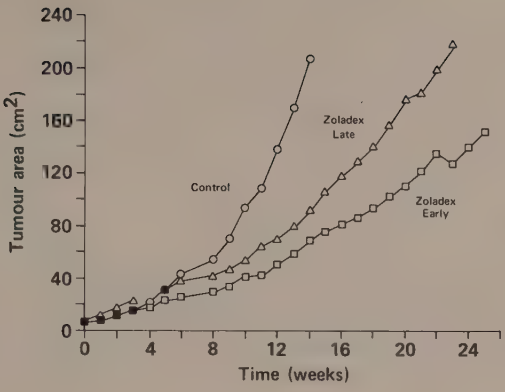

FIGURE 7 Effect of single depots containing 1mg 'Zoladex' given every 28 days on growth of Dunning prostate tumours in rats. Control rats were untreated; early 'Zoladex treatment started 28 days after tumour implantation; late 'Zoladex' treatment started when tumours measured $2cm^2$. There were 14 rats in the control group and 13 rats in each 'Zoladex' treated group

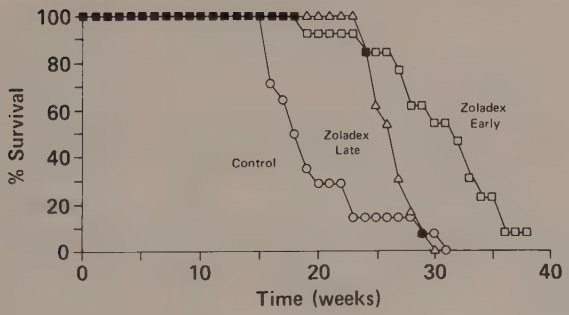

FIGURE 8 Effect of single depots containing 1mg 'Zoladex' given every 28 days on survival of rats with Dunning prostate tumours. Control rats were untreated; early 'Zoladex' treatment started 28 days after tumour implantation: late 'Zoladex' treatment started when tumours measured $2cm^2$. There were 14 rats in the control group and 13 rats in each 'Zoladex' treated group

CONCLUSIONS

It is concluded that LHRH analogues can be effectively administered in a variety of formulations. Biocompatible, biodegradable depots of LHRH agonists are effective, well tolerated and convenient to administer and will find utility in the chronic treatment of sex hormone-responsive benign and malignant diseases.

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